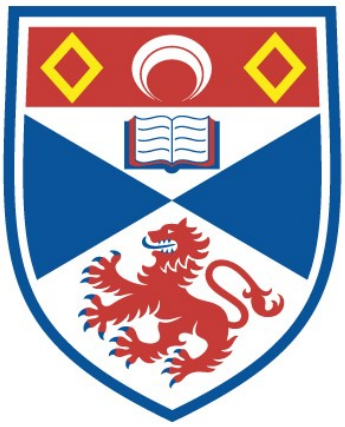
CONFORMATIONAL STUDIES OF FIBRINOGEN AND ITS DERIVATIVES

Angela Apap-Bologna

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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CONFORMATIONAL STUDIES OF FIBRINOGEN AND ITS DERIVATIVES

A THESIS PRESENTED BY

ANGELA APAP-BOLOGNA

TO

THE UNIVERSITY OF ST. ANDREWS
IN APPLICATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT

There is much controversy regarding the conformation of fibrinogen. Several models have been proposed ranging from a trinodular arrangement to a globular conformation. It has also been suggested that fibrinogen has a flexible structure where the shape of the molecule is influenced by its environment, one major factor being calcium concentration. In addition, although the importance of tightly bound calcium ions ($k_d \simeq 1\mu M$) to the fibrinogen molecule is well established, the role of the larger number of low affinity sites ($k_d \simeq 1mM$) is still a matter of some debate.

In this study, the two techniques of photosensitized radioactive surface labelling and photosensitized cross-linking were selected for development and assessment. This was done with a view to examining the conformation of fibrinogen in its native state and under different solvent conditions, with particular reference to the influence of calcium.

The two techniques have been shown to have definite applications in their use as probes into the conformation of fibrinogen. Results derived using these methods support the view put forward by various authors that fibrinogen is a dynamic molecule having a flexible conformation and that the conformation adopted is dependent on solvent composition. Calcium concentration in the millimolar range is particularly significant and consequently so is the saturation of the low affinity binding sites which may well have a regulatory function. Experimentally two extreme conformations have been demonstrated, - a closed, compact structure at low calcium concentrations compared to a more open one at higher calcium concentrations. More importantly, the techniques used also show the subtle changes which occur within the molecule as the calcium concentration is raised, changes which may be more significant physiologically. The most important of these are the effect of calcium on the C-terminal parts of the Aα-chains and the D domains of the molecule.

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that it is my own composition and that no part of it has been previously presented for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, United College of St. Salvador and St. Leonard, University of St. Andrews, under the direction of Dr. Graham D. Kemp.

CERTIFICATE

I hereby certify that Angela Apap-Bologna has spent nine terms engaged in research work under my direction and she has fulfilled the conditions of Ordinance General No. 12, and Resolution of The University Court 1967, No. 1 and that she is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

This work is dedicated to my mother, Elizabeth.

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CONTENTS

TITLE		PAGE
Chapter 1:	INTRODUCTION	
	Part (1): FIBRINOGEN AND ITS DERIVATIVES	
	The chemistry of fibrinogen	1
	Fibrinogen in coagulation	5
	The digestion of fibrinogen	10
	Calcium and fibrinogen	16
	The shape of fibrinogen	22
	Part (2): PHOTOSENSITIZED RADIOACTIVE LABELLING AND PHOTOSENSITIZED CROSS-LINKING	
	Photooxidation techniques	23
	Mechanisms	25
	Part (3): AIMS OF THIS STUDY	30
Chapter 2:	MATERIALS AND METHODS	
	Section (A): PREPARATIVE TECHNIQUES	
	The purification of fibrinogen	31
	The purification of plasminogen	33
	Plasminogen activation	35
	Plasminogen and plasmin assays	35
	The preparation of fragments D and E	38
	The digestion of photolabelled or cross-linked fibrinogen	39
	The purification of fluorescein	40
	Section (B): ANALYTICAL TECHNIQUES	
	Polyacrylamide gel electrophoresis	41
	Laemmli (discontinuous) gels	46
	Detection of protein	48
	Section (C): EXPERIMENTAL TECHNIQUES	
	Photosensitized radioactive labelling	49
	Photosensitized cross-linking	50
Chapter 3:	RESULTS	
	Section (1): PURIFICATIONS	
	Fibrinogen	52
	Fragments D and E	53

Chapter 3: RESULTS [CONT/-] PAGE

Section (2): DEVELOPMENT AND OPTIMIZATION OF TECHNIQUES
Light source 56
Removal of fluorescein and excess [^{3}H]tryptophan 58
Irradiation time 61

Section (3): BUFFER EFFECTS 64

Section (4): CALCIUM STUDIES
Photosensitized cross-linking 73
Photosensitized radioactive labelling 75
Magnesium and sodium chloride studies 80

Chapter 4: DISCUSSION 84

Chapter 5: BIBLIOGRAPHY 129

ILLUSTRATIONS

CHAPTER 1: INTRODUCTION

Figure 1-1 The coagulation cascade.

Table 1-(i) Some physicochemical properties of human fibrinogen. (Doolittle, 1981).

Figure 1-2 Schematic model of a fibrinogen molecule. (Gaffney, 1977).

Figure 1-3 Complete amino-acid sequence of the Aα, Bβ, and γ-chains of human fibrinogen. (Furlan, 1984).

Table 1-(ii) Summary of the known major variants of human plasma fibrinogen chains. (Mosesson, 1983).

Figure 1-4(a)(b) The polymerization model assuming face-to-face attachment of interacting fibrin monomers. (Fowler et al, 1981).

Figure 1-5 Scheme of fibrinogen digestion by plasmin. (Latallo, 1973).

Table 1-(iii) Distribution of calcium (mmol/L) in normal human plasma. (From: Ganong's Review of Medical Physiology, 1983).

Table 1-(iv) Proposed models of fibrinogen conformation.

Figure 1-6 Type I and II sensitized photooxidation mechanisms.

Figure 1-7 Electronic energy states.

Figure 1-8 Dye-sensitized radioactive photolabelling and cross-linking.

CHAPTER 2: MATERIALS AND METHODS

Figure 2-1 The purification of fibrinogen.

Table 2-(i) Peak analysis in the purification of fibrinogen.

Figure 2-2 Elution profile for the first stage of plasminogen purification on lysine-Sepharose.

Figure 2-3 Elution profile for the final stage of plasminogen purification on Sephadex G-25.

Figure 2-4 Plasminogen and plasmin activity assays.

Table 2-(ii) Final concentration of components for SDS-polyacrylamide gel polymerization.

Table 2-(iii) Final concentration of components required for the polymerization of 3% stacking gels and 7½% seperating gels.

Table 2-(iv) $E_{1\%}$ values (280nm) of some proteins used in this study.

Figure 2-5 Experimental protocol.

Figure 2-6 Apparatus used for sample irradiation.

CHAPTER 3: RESULTS

Figure 3-1 Elution profile for the purification of fibrinogen on DEAE-cellulose.

Figure 3-2 Purified fibrinogen after reduction. (Analysed on Laemmli, 3:7½% gels).

Figure 3-3 SDS-polyacrylamide gels of reduced (5% acrylamide) and non-reduced (3% acrylamide) fibrinogen.

Figure 3-4 The digestion of fibrinogen to fragments D and E. (Photograph of 5% acrylamide gel).

Figure 3-5 Elution profile for the seperation of fragments D and E on DEAE-cellulose.

Table 3-(i) Dependence of radioactive incorporation on light source used.

Figure 3-6 SDS-polyacrylamide gels (5% acrylamide) of reduced fibrinogen, before and after dialysis.

Table 3-(ii) The removal of excess [^{3}H]tryptophan by dialysis.

Figure 3-7(a)(b) Densitometer scans of SDS-polyacrylamide gels (3% acrylamide) of fibrinogen samples irradiated for (a) 30 seconds and (b) 60 seconds respectively.

Table 3-(iii) Photosensitized radioactive labelling at 30 seconds and 60 seconds.

Figure 3-8 Graph of residual radioactivity in dialysed samples (dpm x 10^{-3}) versus irradiation time (seconds).

Figure 3-9 SDS-polyacrylamide gels (3% acrylamide) of fibrinogen irradiated for 10 - 60 seconds in 0.05M Tris-HCl, pH 7.5, containing 2mM $CaCl_2$.

Figure 3-10	Graphs of (a) % polymer and (b) % monomer versus irradiation time (seconds).
Figure 3-11	Graph of radioactive incorporation (dpm/pmole fibrinogen x 10^{-2}) versus irradiation time (seconds).
Figure 3-12(a)(b)	SDS-polyacrylamide gels (3% and 5% acrylamide respectively) of fibrinogen irradiated for 0 - 60 seconds in 0.13M NaCl, 6.7.mM sodium phosphate, pH 7.0.
Figure 3-13	Graph of radioactive incorporation (dpm/pmole fibrinogen x 10^{-2}) versus irradiation time (seconds).
Table 3-(iv)	Incorporation of radioactive label into fragment E.
Table 3-(v)	Incorporation of radioactive label into fragment E at pH 7.5.
Table 3-(vi)	Radioactive incorporation into fragment E at pH 7.0, 7.5, and 8.0, in 8M urea.
Figure 3-14	Graph of radioactive incorporation (dpm x 10^{-3}) versus distance from top of gel (cms). (Acrylamide concentration = 5%).
Table 3-(vii)	The photosensitized radioactive labelling of intact fibrinogen.
Table 3-(viii)	Radioactive labelling of the Aα, Bβ, and γ-chains of fibrinogen.
Figure 3-15(a)(b) and (c)	Gels of reduced, (a) and (b), and non-reduced, (c), fibrinogen samples following cross-linking under different solvent conditions.
Figure 3-16(a)(b)	SDS-polyacrylamide gels (3% and 5% acrylamide respectively) of fibrinogen cross-linked at various calcium concentrations.
Figure 3-17	Intermolecular cross-linking of fibrinogen as a function of calcium concentration.
Figure 3-18	The surface labelling of fibrinogen as a function of calcium concentration.
Figure 3-19	Incorporation of label into the D and E domains as a function of calcium concentration.
Figure 3-20	Relationship between E accessibility (ie. label in E domain; dpm/pmole protein) and % uncross-linked Aα-chain.

Figure 3-21 Graph of the incorporation into fibrinogen and the sum of the incorporation into the core domains, as a function of calcium concentration.

Figure 3-22 Comparison of the incorporation of label into the D domain and fragment D as a function of calcium concentration.

Figure 3-23 Graph of incorporation of label into the Aα chains as a function of calcium concentration.

Figure 3-24 Comparison of the incorporation of label into the D domain and the Aα chains as a function of calcium concentration.

Figure 3-25 Comparison of the incorporation of label into the E domain and fragment E as a function of calcium concentration.

Table 3-(ix) Incorporation of radioactive label into the E domain and fragment E.

Figure 3-26(a)(b) SDS-polyacrylamide gels (3% and 5% acrylamide respectively) of fibrinogen cross-linked at various magnesium concentrations.

Figure 3-27 Comparison of the effect of calcium / magnesium on intermolecular cross-linking.

Figure 3-28(a)(b) SDS-polyacrylamide gels (3% and 5% acrylamide respectively) of fibrinogen cross-linked at various sodium chloride concentrations.

Figure 3-29 Comparison of the effect of calcium / magnesium on the photosensitized radioactive labelling of fibrinogen.

Figure 3-30 Comparison of the effect of calcium / sodium chloride on the photosensitized radioactive labelling of fibrinogen.

CHAPTER FOUR: DISCUSSION

Figure 4-1 Proposed models of fibrinogen conformation by Hall and Slayter (1959), and Koppel (1966).

Figure 4-2 Updated schematic depiction of the vertebrate fibrinogen molecule postulated by Doolittle et al (1977).
From: Doolittle (1983).

Figure 4-3 Different possible forms of the fibrinogen molecule proposed by Hudry-Clergeon et al (1975).

Figure 4-4 Schematic model of the fibrinogen molecule postulated by Mosesson et al (1981).

Figure 4-5 Different isomeric structures of fibrinogen suggested by Mueller and Burchard (1978).

Figure 4-6 The role of the α-C domains in fibrin assembly. (Medved' et al, 1986).

ABBREVIATIONS

A = absorbance

ADP = adenosine 5'-diphosphate

bisacrylamide = N,N'-bis-methylene acrylamide

C-terminal = carboxyl terminal

DEAE-cellulose = diethylaminoethyl groups covalently linked to a cellulose matrix

dpm = disintegrations per minute

EACA = ϵ-amino caproic acid

EDTA = ethylene diamine tetraacetic acid

EGTA = ethyleneglycol bis-(β-aminoethylether)N,N'-tetracetic acid

[^{3}H]trp = tritiated tryptophan

MW = molecular weight

PAGE = polyacrylamide gel electrophoresis

rpm = revolutions per minute

SDS = sodium dodecyl sulphate

TEMED = N,N,N',N'-tetramethylethylene diamine

Tris = 2-amino-2-(hydroxymethyl)propane-1,3-diol

CHAPTER ONE

INTRODUCTION

Part (1): Fibrinogen and Its Derivatives

Fibrinogen is a soluble plasma glycoprotein and the precursor of fibrin monomers which in turn polymerize to form insoluble fibrin clots (see Figure 1-1). The protein thus forms part of a finely regulated system, the disruption of which results in various clinical conditions such as thrombosis and coronary artery disease. Consequently any advancement in the prevention and treatment of such diseases depends on the clarification of the various complex mechanisms of haemostasis.

Since fibrinogen plays a central role in blood coagulation, the understanding of the biochemistry of this protein has been avidly sought over the years (see eg., reviews by Doolittle, 1981, 1984). Prior to stating the aims of this work, the main aspects of such research carried out on the fibrinogen molecule will be reviewed.

The Chemistry of Fibrinogen

Fibrinogen is synthesized by the parenchymal cells of the liver and its molecular weight is approximately 340,000 ± 5%. (As determined by analytical ultracentrifugation (Shulman, 1953; Caspary and Kekwick, 1957;) and light scattering (Katz et al,

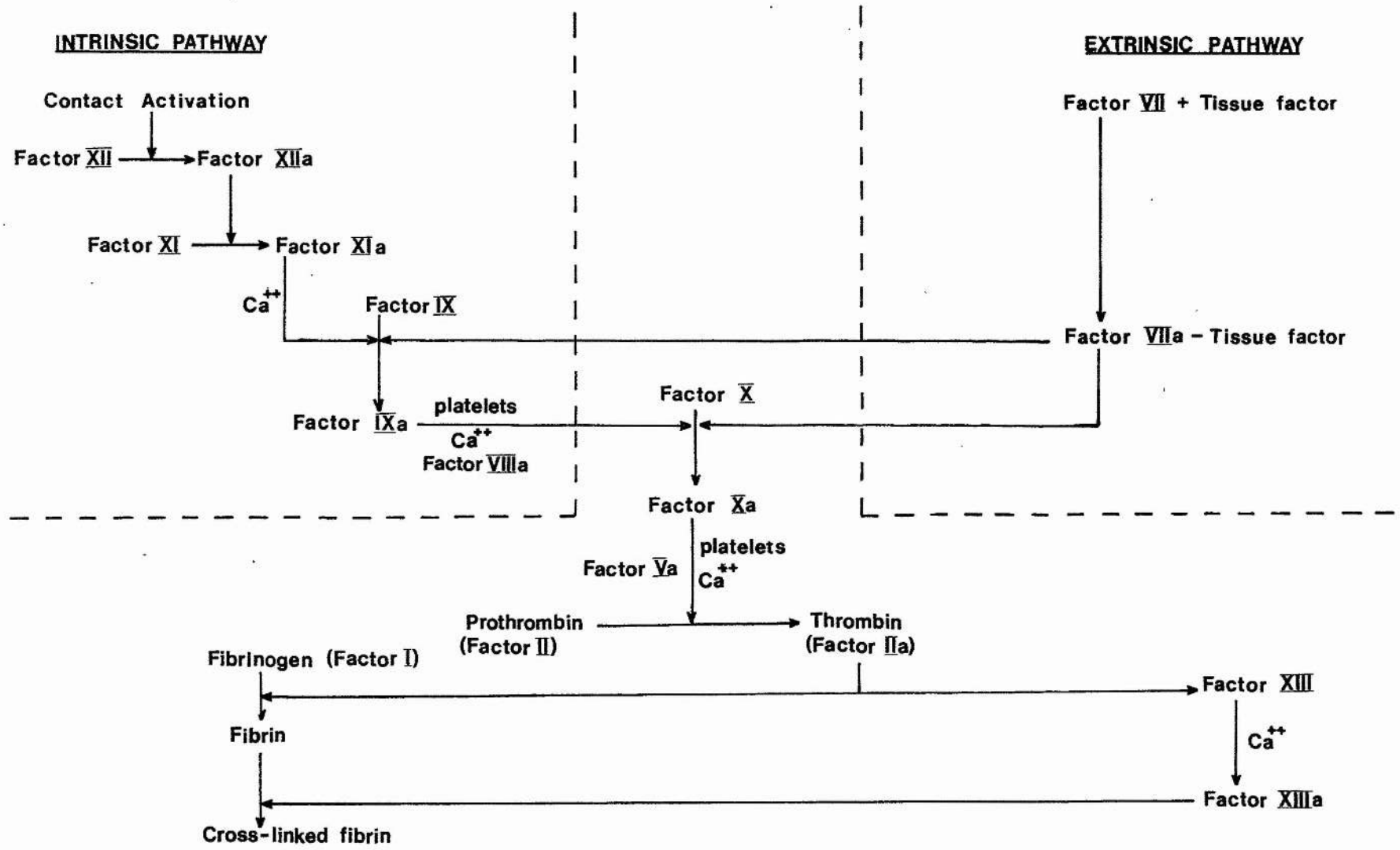


Figure 1-1: The coagulation cascade.
(Adapted from: Shuman and Greenberg, 1986).

1952)). Human fibrinogen is a glycoprotein, containing about 3% carbohydrate. It constitutes 5% of the plasma proteins and the normal concentration found in human plasma is 9μM (or 3mg/ml). Fibrinogen differs from most other plasma proteins by its reduced solubility at low temperatures. Some of the physicochemical properties of human fibrinogen can be seen in Table 1-(i) (Doolittle, 1981).

As can be seen from the schematic model of fibrinogen in Figure 1-2 (Gaffney, 1977), the molecule has a chemically symmetrical structure and is composed of three pairs of non-identical chains, Aα, Bβ, and γ. The chains are all connected and held together by disulphide bonds to give a dimeric subunit formula of ($A\alpha_2B\beta_2\gamma_2$) (Clegg and Bailey, 1962). The constituent reduced chains have been shown (by SDS-polyacrylamide gel electrophoresis) to have molecular weights of 67,000 (Aα), 58,000 (Bβ), and 47,000 (γ) (Gaffney and Dobos, 1971).

Full analysis of the primary structure of fibrinogen has been carried out and the complete sequence of each chain is known, ie. Aα (Doolittle et al, 1979), Bβ (Henschen and Lottspeich, 1977; Watt et al, 1979), and γ (Lottspeich and Henschen, 1977); the chains contain 610, 461 and 411 residues respectively (see Figure 1-3). Each of the three chains have certain distinct characteristics which are reflected in their amino-acid sequences. However, extensive homology has also been observed between the three non-identical chains, particulary

Table 1-(i) : Some Physicochemical Properties of Human Fibrinogen. (Doolittle, 1981)

Molecular weight	340,000
Sedimentation coefficient ($S_{20,w}$)	7.9S
Translational diffusion coefficient ($D_{20,w}$)	20 x 10^{-7} cm^2 s^{-1}
Frictional ratio (f/f_o)	2.34
Partial specific volume ($\bar{v}$)	0.715
Intrinsic viscosity ($\|\eta\|$)	0.25 dl/g
Per cent α-helix	33
Isoelectric point (at ionic strength = 0.2)	5.5
Extinction (or absorbance) coefficient ($E^{1\%}_{1cm,280}$)	15.1 - 15.5

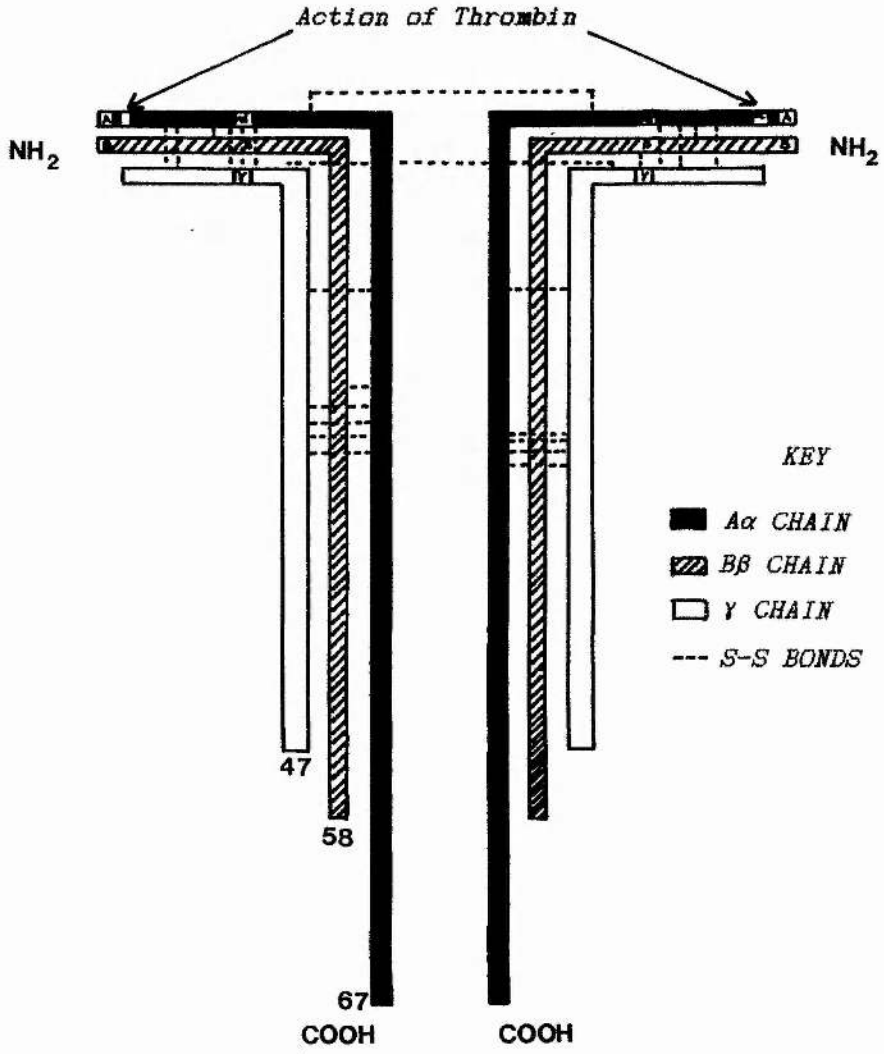
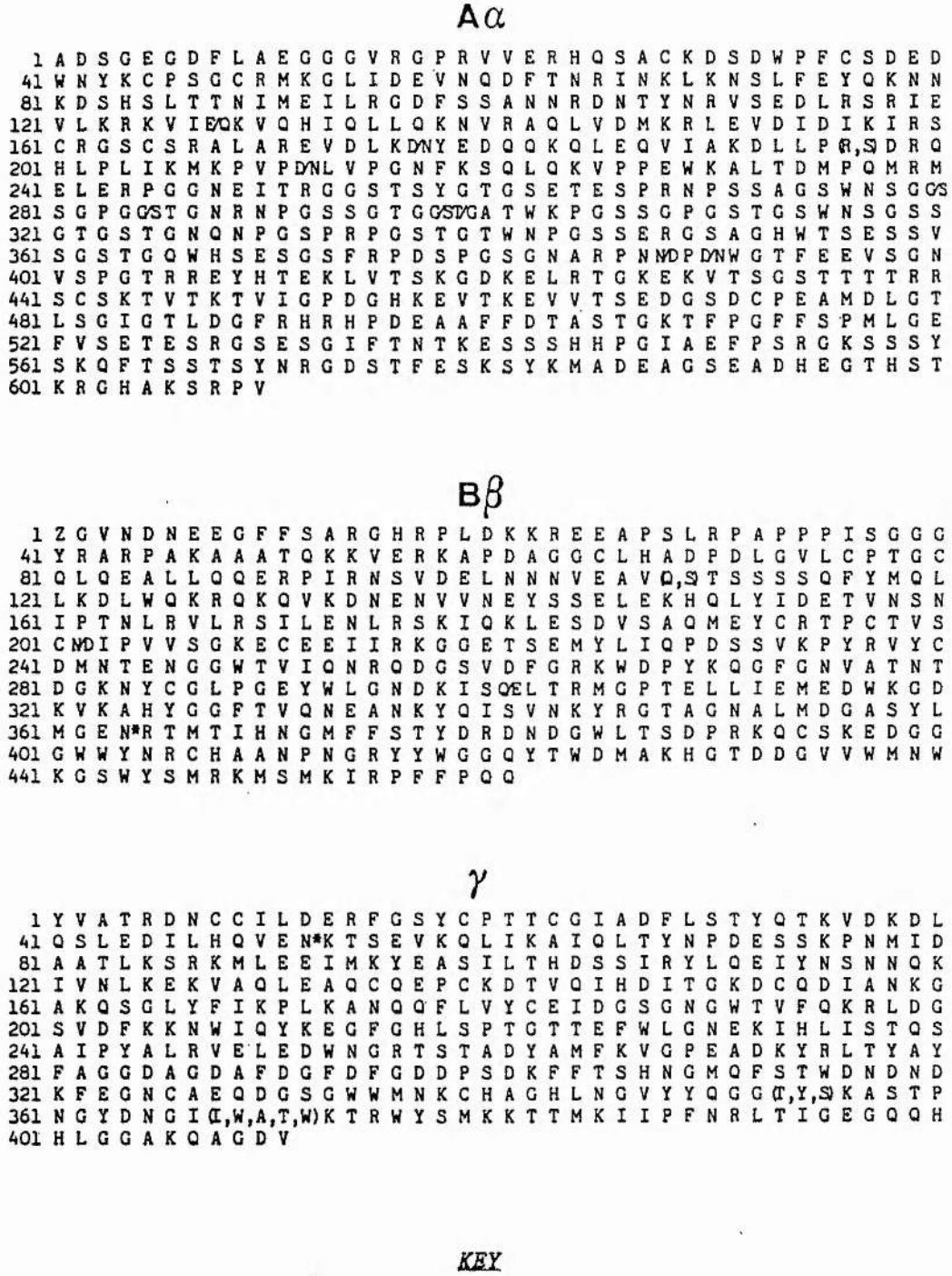


Figure 1-2: Schematic model of a fibrinogen molecule. (From: Gaffney, 1977).



KEY

A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile,
K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser,
T = Thr, V = Val, W = Trp, Y = Tyr, Z = pyrrolidonecarboxylic acid,
* = site of carbohydrate attachment.

Figure 1-3: Complete amino-acid sequence of the Aα, Bβ, and γ chains of human fibrinogen. (From: Furlan, 1984).

between the Bβ and γ-chains. It has thus been suggested (Doolittle, 1976) that all three chains are descended from a common ancestor. These similarities and differences bear out on the overall structural features of the fibrinogen molecule.

As seen earlier (Figure 1-2), disulphide bonds are very important with respect to the structural integrity of the molecule. There are 58 cysteine residues in human fibrinogen (Henschen, 1964), but no free SH-groups (Loewy et al, 1961). It was thus concluded that all these residues are involved in disulphide bridges (Henschen, 1964; Henschen and Blomback 1964;). Of the 29 disulphide bridges, three are involved in linking the dimeric halves of the fibrinogen molecule together (Blomback et al, 1976), seven connect individual subunit polypeptides within each half of the molecule, and the remaining disulphide bridges form intrachain bonds (Gardlund et al, 1977; Henschen, 1978;). The cysteine residues have been found to be concentrated in three clusters along each polypeptide chain (as illustrated by Henschen et al, 1982). The amino terminal of the molecule is held tightly together in a dimeric form and this is known as the N-terminal disulphide knot (ie. N-DSK or Hi1-DSK, obtained from cyanogen bromide cleavage of human fibrinogen). The latter was found to contain all three inter-dimeric half bridges, namely one disulphide bond between the two Aα-chains and two disulphide bonds between the γ-chains (Gardlund et al, 1977).

It is well established that human fibrinogen contains four heterogenous carbohydrate side chains, namely the neutral hexoses, mannose and galactose, the hexosamine, N-acetyl-glucosamine, and sialic acid as the charged, terminal, carbohydrate (see review by Haberli, 1984). Each Bβ and γ-chain carries one sugar side chain, N-glycosidically linked to asparagine residue number 364 of the Bβ-chain (Topfer-Petersen et al, 1976) and number 52 of the γ-chain (Kowalska-Loth et al, 1973). The role of the carbohydrate moieties is not very clear, however various authors have shown that the removal of sialic acid enhances the clotting of fibrinogen (Chandrasekhar et al, 1962; Martinez et al, 1977, 1978;).

In addition to its other properties, fibrinogen also exhibits a certain degree of molecular heterogeneity which can be ascribed to a variety of causes (see Table 1-(ii)). Investigation of plasma fibrinogen subfractions (I-1 to I-9) of varying solubility (Mosesson and Sherry, 1966) have led to the recognition that a major proportion of circulating fibrinogen is composed of coagulable species that have undergone varying degrees of proteolytic attack resulting in the cleavage and release of C-terminal Aα-chain peptides (Mosesson et al, 1972a). In addition, three major types of Aα-chains have been identified that differ with respect to their fibrinopeptide A composition (Blomback et al, 1966). One such type (Y) exhibits deletion of the amino-terminal alanine, whilst another type (AP) contains a phosphorylated serine residue in position 3. Heterogeneity of

Table 1-(ii) : Summary of the Known Major Variants of Human Plasma Fibrinogen Chains. (Mosesson, 1983).

Terminology	Variation	Essential structural difference	Comment
A	Fibrinopeptide A structure	-	
AP		phosphorylated serine	unknown functional difference
Y		lacks NH_2-Ala	
Aα/2, Aα/4 Aα/6...etc;	Aα/chain size	lack of peptides containing C-terminal regions due to proteolysis and release from parent molecules	loss of peptides results in delay in fibrin aggregation rate
$B\beta_L$	Bβ chain carbohydrate content (sialic acid)	2 sialic acid residues/chain	desialation enhances fibrin aggregation rate
$B\beta_R$		1 sialic acid residue/chain	
γ-1	γ chain carbohrdrate content (sialic acid)	2 sialic acid residues/chain	
γ-2		1 sialic acid residue/chain	
γ	γ chain amino acid sequence (chain size)	C-terminal sequences differ	unknown functional difference
γ'		γ' larger than γ	

the Bβ (ie. $B\beta_L$ and $B\beta_R$) and γ (ie. γ-1 and γ-2) chains has been observed regarding the sialic acid content of their carbohydrate moiety (Gati and Straub, 1978). As well as the latter differences in the γ-chain, another class of functionally equivalent γ-chains was reported (ie. γ') by Mosesson et al, (1972b). The γ' chain is longer that that from the major population of γ-chains by virtue of amino-acid sequence differences in the respective C-termini.

Fibrinogen in Coagulation

As seen earlier (Figure 1-1) fibrinogen plays a major role in the coagulation cascade and is the natural substrate for thrombin, the final proteolytic enzyme in this chain of events. By cleaving only about 1% of the mass of fibrinogen, thrombin initiates a self-assembly process and the formation of an insoluble fibrin clot.

In their review regarding the structure and interaction of fibrin, Hermans and McDonagh (1982) divide the process of fibrin assembly and cross-linking into four steps, namely :

(1) Activation of fibrinogen by thrombin,
(2) End-to-end polymerization of monomeric fibrin to form protofibrils,
(3) Lateral association of protofibrils to fibrin fibres,
(4) Covalent cross-linking of fibrin by factor XIIIa.

Each of these stages will now be discussed in further detail.

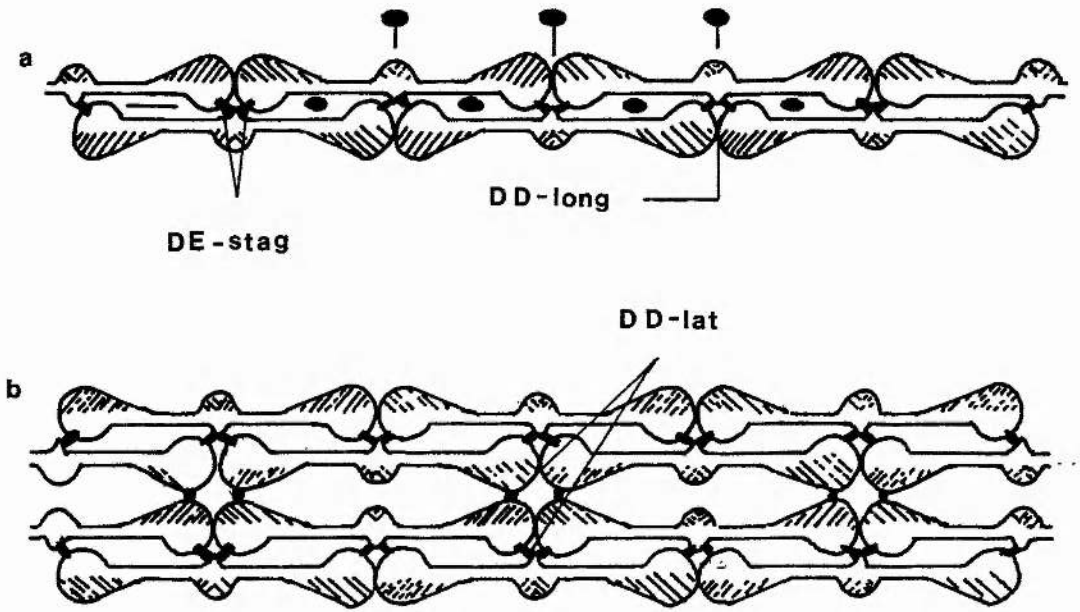
Thrombin has been shown to proteolytically attack the Aα and Bβ-chain pairs of fibrinogen, thus releasing four negatively charged peptides from the molecule, termed fibrinopeptide A (from each Aα-chain) and fibrinopeptide B (from each Bβ-chain) respectively (Bailey et al, 1951; Lorand, 1951;). Enzymic attack occurs at the N-termini of the chains, at two sets of Arg-Gly bonds, ie. (Aα 16-17 and Bβ 14-15) and the resulting thrombin-modified molecules are denoted as fibrin monomers. (The modified chains of these species are termed α, β and γ). Fibrinopeptide A is released faster than the B peptide (Blomback and Blomback, 1972) and two molecular models have been proposed to explain this difference in cleavage rates, - a sequential and a competitive model. According to the former mechanism, cleavage of the A peptide from fibrinogen initiates ordered alignment of desA-fibrin monomers which in turn induces a specific conformational change in the fibrinogen molecule. This then results in an increased susceptibility of the Bβ peptide bond to thrombin action (Blomback et al, 1978). In contrast, the competitive model assumes full access of thrombin to the Aα and Bβ-chain cleavage sites, however in this mechanism it is suggested that peptide A is released faster than peptide B because the rate constant for the hydrolysis of the specific Arg-Gly bond in the Aα-chain is greater than that for the Bβ-chain (Martinelli and Scheraga, 1980). It has also been shown that desA-fibrin monomers (prepared by use of the snake venom

reptilase which only releases the A peptide) are sufficient for the initiation of fibrin assembly (Blomback and Blomback, 1972).

As well as reducing the excess negative charge on fibrinogen molecules, the release of fibrinopeptides exposes definite contact sites which are masked in intact fibrinogen. Polymerization of the fibrin monomer units then occurs spontaneously (Ferry, 1952) to form a non-covalently bonded gel (steps (2) and (3)), and the polymer can then be covalently cross-linked by a calcium-dependent enzyme, factor XIIIa, itself activated by thrombin (step 4).

The first step in this process involves the end-to-end polymerization of monomeric fibrin to long strands two molecules in diameter which are called protofibrils (Fowler et al, 1981; see figure 1-4(a)). The contacts that hold the monomers together are: (a) longitudinal, between the terminal D nodules of fibrinogen (ie. DD-long contacts), and (b) staggered, lateral contacts between the terminal and the central E nodule (ie. DE-stag contacts). The model shown in figure 1-4(a) assumes 'face-to-face' attachment of interacting fibrin monomers and accomodates the observation that two-stranded protofibrils are formed in the course of fibrin polymerization as a distinct structural intermediate.

Thus both pairs of complementary contact sites are saturated within a double stranded protofibril and the latter is closed



a = Two-stranded protofibril.
b = Lateral association of protofibrils to make the fibrin fibre.

Figure 1-4: The polymerization model assuming face-to-face attachment of interacting fibrin monomers.
(From: Fowler et al, 1981).

with respect to lateral growth. The existence of another intermolecular contact site, ie. DD-lat (see Figure 1-4(b)) was thus postulated and involves the terminal D domains. These contacts are responsible for lateral association (step 3) of protofibrils to fibres. The latter form as a network and consequently the final fibrin solution is a gel. The binding affinity of the DD-lat polymerization sites is weak, the latter being deduced from the observation that protofibrils do not begin to form fibres until they have grown to exceed some critical length. The existence of this contact site is still speculative and Erickson and Fowler (1983) suggest an alternative possibility, ie. that adjacent protofibrils could also be 'staggered' (and not in register as in Figure 1-4(b)), in which case additional lateral contacts between the D and E nodules (ie. DE-lat) could also occur.

Alternative models for fibrin polymerization have also been put forward (Olexa and Budzynski, 1980). The latter workers assume 'face-to-back' alignment of fibrin monomers and all laterally packed fibrin strands face the same direction. Thus, by this mechanism, the two-stranded protofibril would not be a distinct structural intermediate because a third and succeeding strands could be added using the same DE-stag contacts which are always exposed on the free edge. This type of 'open' polymerization is not compatible with the evidence put forward by various authors, showing the two-stranded protofibrils to be the predominant polymer species at early times of assembly

(Ferry, 1952; Krakow et al, 1972; Hantgan and Hermans, 1979; Hantgan et al, 1980;).

The final stage in fibrin formation is the cross-linking of fibrin via the formation of isopeptide bonds catalysed by a calcium-dependent enzyme, factor XIIIa (Lorand, 1972).

However before this can be achieved, generation of the active enzyme from the fibrin stabilizing factor zymogen (factor XIII or a_2b_2) must be accomplished by unmasking of the active centre residue cysteine on the catalytic subunit. This occurs in two distinct stages and requires both thrombin and calcium ions (Lorand and Konishi, 1964). First thrombin removes an amino-terminal peptide fragment from the 'a' subunits of the a_2b_2 zymogen. This, however, still leaves the subunits heterologously associated as $a_2'b_2$ (ie. factor XIII') which is not sufficient for generating transamidase activity. Expression of the latter is dependent on calcium ions which cause the release of non-catalytic b subunits (Curtis et al, 1974), as well as the conversion of the a_2' to a_2^*. The latter represent the functionally competent enzyme, factor XIIIa.

Factor XIIIa then forms intermolecular γ-glutaminyl-ϵ-lysyl cross-links between fibrin molecules. Only selected lysine and glutamine residues can take part in the cross-linking reaction and up to six isopeptide bonds may be introduced per mole of fibrin monomer under optimum conditions.

A pair of cross-links is first formed between the C-terminal portions of the γ-chains of adjacent fibrin molecules to form γ-γ dimer (Chen and Doolittle, 1971). Thus a glutamine (residue 397) and a lysine (residue 405) on one γ-chain undergo reciprocal cross-linking with those from another γ-chain.

During prolonged action of factor XIIIa on fibrin, α-chains also become cross-linked to other α-chains, leading to the formation of α-chain multimers (McKee et al, 1970). There may be 2-4 potential cross-links distributed among the α-chains and it has been suggested that the α-chain cross-link sites are located in the central portion of this chain (Fretto et al, 1978). In fact, Doolittle et al (1979) showed that specific α-chain glutamine residues incorporate radioactive substitute donors (ie. ^{14}C-glycine ethyl ester) under the influence of factor XIIIa, namely residues 328 and 366 in the middle region of the α-chain.

The Digestion of Fibrinogen

Although the formation of blood clots is essential, a mechanism for their removal when they are no longer needed or for dissolving any fibrin in the general circulation is also important. Thus a fine biochemical balance must be struck in order to maintain intravascular fluidity while simultaneously retaining the capacity to seal any leaks in the system.

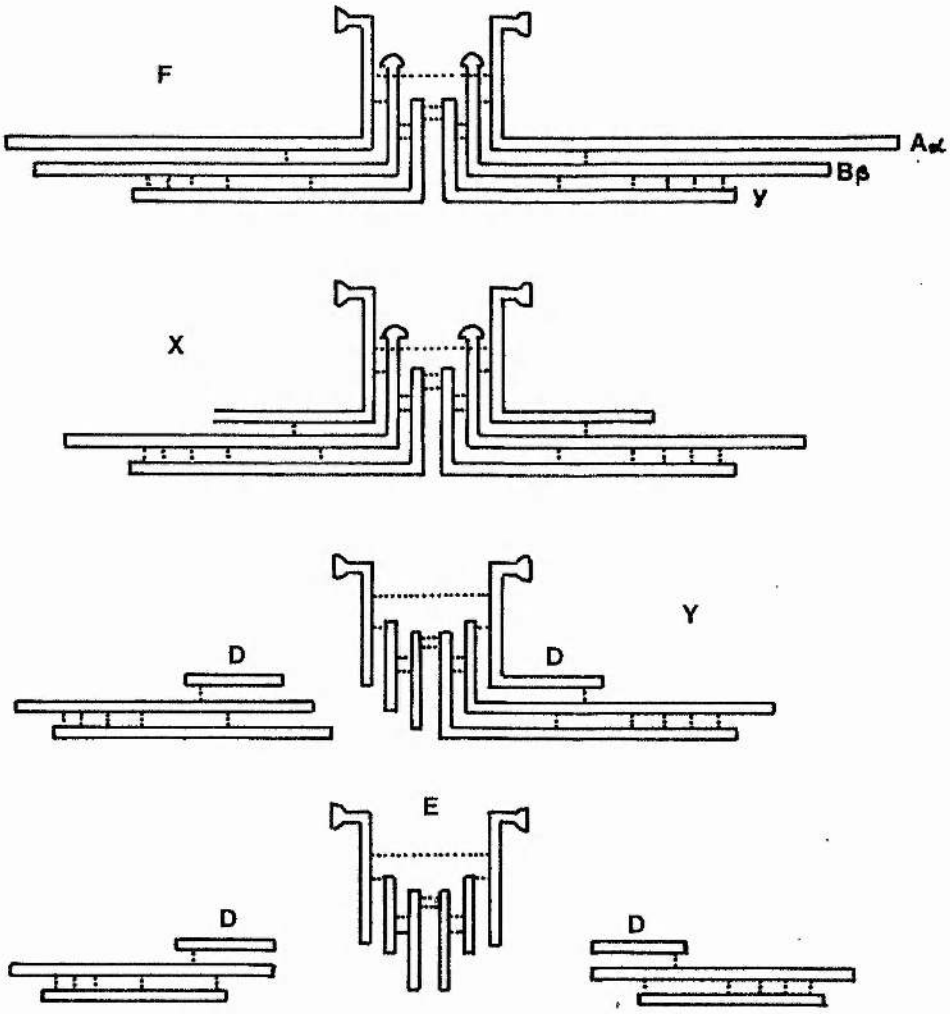
Plasmin (molecular weight : 85,000, derived from the zymogen, plasminogen,) is the key enzyme in the fibrinolytic system and has a directly opposite effect to that of thrombin. The conversion of plasminogen to plasmin (see review by Kosow, 1976) may be catalyzed by various proteases such as those found in many tissues (tissue activators), in blood (blood/vascular activators), or body fluids eg. urine (urokinase). Various strains of streptococci produce the extracellular protein, streptokinase, which is also capable of activating human plasminogen. The zymogen may also undergo spontaneous activation which is accelerated by glycerol, di- and tri- ethylene glycols. Although both plasmin and thrombin are serine proteases, they act on the same substrate, fibrinogen, with remarkably different specificities. In effect thrombin promotes the construction of blood clots whilst plasmin plays a key role in their removal.

Plasmin preferentially catalyzes the hydrolysis of peptide bonds between the carboxyl-group of lysine or arginine and the amino-group of another amino acid. The interaction between fibrinogen and plasmin was first studied by Nussenzweig (1961) who used DEAE-cellulose column chromatography to seperate five 'final' fibrinogen degradation products after prolonged lysis by the enzyme. The author called these fractions A, B, C, D and E. However, on the basis of column chromatography and gel electrophoresis studies, Marder et al (1969) proposed an asymmetric cleavage model of fibrinogen degradation, the main factors of which are generally accepted to date. Figure 1-5

shows the major aspects of this sequence of degradation reactions and the products produced.

Breakdown commences by the degradation of the polar C-terminals of the Aα-chains and the products of this initial step are denoted as fragments X. The latter represent a mixture of several molecular species in which the C-terminal two-thirds of the Aα-chains have been degraded to various extents, followed by sequential degradation at the N-terminals of the Bβ-chains in 'late' fragment X species. Molecular weights of the X fragments vary from 240,000 to 337,000 (Furlan and Beck, 1972; Liu et al, 1986;). Cottrell and Doolittle (1976) indicated that the earliest plasmic cleavage occurs at Aα 583 (lysine) - 584 (methionine), with the release of a peptide (molecular weight: 2,859) containing the C-terminal 27 amino-acid residues of the Aα-chain. Other sites of plasmic cleavage on the latter chain exist at Aα 424-425 and Aα 206-207, whilst cleavage of the Bβ-chain occurs at Bβ 42-43 (Liu et al, 1986).

Next, asymmetric interdomainal attack in the centre of one of the three stranded ropes of fragment X occurs. The result is fragment Y (molecular weight : 150,000-160,000) and fragment D (molecular weight : 70,000-95,000). Further interdomainal cleavage of fragment Y then occurs and this results in a second fragment D and another digest product, fragment E (molecular weight : 50,000-60,000). Thus, during extensive plasmin



F = fibrinogen
X = fragment X
Y = fragment Y
D = fragment D
E = fragment E

Figure 1-5: Scheme of fibrinogen digestion by plasmin. (Latallo, 1973).

digestion, two moles of the 'core' fragment D and one mole of 'core' fragment E are produced from one mole of fibrinogen.

Gaffney (1973) showed that the end-products of digestion of non-crosslinked fibrin are electrophoretically indistinguishable from those resulting from the digestion of fibrinogen. However, prolonged lysis of cross-linked fibrin produces an additional major fragment, namely γ-cross-linked D-dimer (Kopec et al, 1973). Fragment E binds to D-dimer with high affinity to form a D-dimer - E complex and the latter has been isolated from the digests of cross-linked fibrin (Hudry-Clergeon et al, 1974).

Three molecular forms of fragment E (ie. E_1, E_2, E_3) have been isolated (Olexa et al, 1981). The fragment corresponds to the central dimeric domain of fibrinogen, sharing all N-termini of the six chains and also corresponds closely to the dimeric disulphide knot (N-DSK) isolated by cyanogen bromide fragmentation of fibrinogen. In their studies on the stimulation of fibrinogen synthesis in cultured rat hepatocytes by fibrinogen and its degradation products, D and E, Qureshi et al (1985) showed that only fragment E was a specific stimulator of fibrinogen biosynthesis. These workers thus suggested that the fragment plays an important role in maintaining normal levels of plasma fibrinogen.

Each fragment D consists of the remnants of the Aα, Bβ and γ-chains of fibrinogen and corresponds to each of the outer

terminal domains of the native molecule. Several fragment D species have been recovered from plasmic digests in the absence of calcium ions (Furlan et al, 1975; Takagi and Doolittle, 1975; Lawrie et al, 1977;). All these species contain the same α and β remnants (molecular weights : 10,000 and 45,000 respectively), however the heterogeneity of the fragment was shown to be caused by the gradual degradation of the γ-chain at its C-terminal end. The γ-chain molecular weight was thus found to vary from 39,000 to 13,000.

It was also shown (Gaffney and Brasher, 1973) that the D-dimer produced by digestion of cross-linked fibrin is comprised of γ-chains corresponding to those found in the earliest forms of fragment D, as distinct from later forms. This information was not only consistent with the positioning of the γ-chain cross-linking site close to the C-terminus of this chain, but also confirmed that lysis by plasmin of fragment D occurred at the C-terminal of the γ-chain.

At a physiological calcium concentration, Haverkate and Timan (1977) showed that only the largest (early) species of fragment D is produced, indicating that calcium ions protect this fragment against further attack by plasmin. The authors designated this fragment D species as D(cate) (molecular weight: 93,000) and found that after removal of calcium ions with EGTA, the fragment is further degraded to a final fragment D (ie. D(EGTA), molecular weight : 80,000), by breakdown of the γ-chain

from 38,000 to 25,000. On the basis of these findings, Haverkate and Timan also suggested that D(cate) represents the physiologically important fragment D type. Haverkate et al (1979) also showed that D(cate) exhibits anti-clotting activity, whereas shortening of the γ-chain remnants (as in D(EGTA)) leads to the loss of such activity. The authors thus concluded that the integrity of this γ-chain remnant (molecular weight: 13,000) is essential for the anti-clotting properties of fibrinogen fragments D.

In 1982 Nieuwenhuizen et al (reference (a)) prepared an additional fragment D, D(int), which had a γ-chain molecular weight of 29,000, but which possessed no anti-clotting properties. Consequently these authors suggested that the anti-clotting properties of D(cate) reside in the γ 9-kDalton stretch that is absent in D(int).

Cross-linking patterns (using the chemical agents, dimethyl suberimidate, dimethyl adipimidate, and tetranitromethane) of fibrinogen fragment D prepared either in the presence of calcium ions or EDTA were shown to be consistently different from each other (Britton et al, 1982). Fragment D(EDTA) showed much intermolecular cross-linking indicating that the loss of the C-terminus of the γ-chain remnant results in this fragment D adopting a more open conformation. Neither the addition of 2M urea or EDTA to fragment D(Ca^{++}) altered its cross-linking pattern, suggesting that the conformational change follows

cleavage of a plasmin-susceptible bond which is normally protected by the presence of calcium ions. These results were supported by further studies from workers in this laboratory (Britton et al, 1983).

Calcium and Fibrinogen

Ionized calcium (Ca^{++}) is the biologically active fraction of total serum calcium, the remainder being bound to plasma proteins and anions (see Table 1-iii). It is this free calcium that is necessary for several basic physiological functions which include blood coagulation. Ionized calcium also plays a principal role in maintaining the structure and physiological properties of fibrinogen (see review by Kemp, 1984).

Ly and Godal (1973) demonstrated that the presence of calcium provided protection of fibrinogen against moderate denaturation by heat or alkali, whilst Komenko and Belitser (1963) showed that calcium protects the molecule against proteolytic degradation by trypsin. In addition, Marguerie (1977) demonstrated that the molecule is more resistant to the initial rate of attack by plasmin in the presence of calcium and also that fibrinogen was more stabilized against temperature and acid denaturation in the presence of this cation.

Table 1-(iii) : Distribution of Calcium (mmol/L) in Normal Plasma.
(From : Ganong's Review of Medical Physiology, 1983).

DIFFUSIBLE		1.34
Ionized calcium (Ca^{++})	1.18	
Complexed to HCO_3^-, citrate, etc.,	0.16	
NON-DIFFUSIBLE (protein-bound)		1.16
Bound to albumin	0.92	
Bound to globulin	0.24	
TOTAL PLASMA CALCIUM		2.50

In their studies regarding the effect of calcium ions on the polymerization of fibrin monomers, Endres and Scheraga (1972) showed that calcium is required as a catalyst for this process. In order to explain this catalytic property, the authors suggested that calcium ions bind to specific binding sites in fibrinogen and the fibrin monomer. Marguerie (1977) proposed that the structural features of fibrinogen are related to the existence of such calcium binding sites in the molecule. The calcium binding properties of fibrinogen were further investigated by Marguerie et al (1977) by means of equilibrium dialysis studies on bovine fibrinogen. These studies revealed that the molecules did indeed possess a number of specific binding sites for calcium. The authors showed that at pH 7.5 fibrinogen has 3 binding sites of high affinity (k_d = 2 x 10^{-6}M) and several (14-20) binding sites of low affinity (k_d = 10^{-3}M). Marguerie and co-workers found that the sites of low affinity were eliminated at 10^{-2}M $MgCl_2$, and thus suggested that these are not specific and are due to weak interactions. The authors also showed that the three sites of high affinity are not identical since one of these sites was eliminated at pH 6.0. Several other investigators have shown that fibrinogen from other species (including human fibrinogen) also contain high and low affinity calcium binding sites (Van Ruijven-Vermeer et al, 1978; Kemp et al, 1983;).

As seen earlier, calcium plays an important role in regulating the digestion of fragment D by plasmin (Haverkate and

Timan, 1977). A number of studies have indicated that each fragment D contains one high affinity calcium binding site (Lawrie and Kemp, 1978; Purves et al, 1978; Nieuwenhuizen et al, 1979;). Purves et al (1978) suggested that calcium ions protect D(cate) by holding down a γ-chain loop, about 100 residues from the C-terminal end of this chain, to the remainder of fragment D, whilst Lawrie and Kemp later (1979) proposed that the calcium binding site is located within the γ-chain, forming an intrachain bridge near the C-terminus of this chain.

Precisely what residues are involved in the calcium binding site on fragment D is still a matter of some debate. D(cate) (γ-chain molecular weight : 38,000), in contrast to D(EGTA) (γ-chain molecular weight : 25,000), is capable of binding one calcium ion per molecule. In their isolation of D(int) (γ-chain molecular weight : 29,000) Nieuwenhuizen et al (1982a) found that this fragment retains its calcium binding properties. The authors thus proposed that the calcium binding site of D(int) (and D(cate)) is directly dependent on the 4-kDalton portion of the γ-chain (residues 303-321/356) which is present in D(int) but not in D(EGTA). This conclusion was supported by the findings of Varadi and Scheraga (1986) who, on the basis of their equilibrium dialysis binding studies, proposed that segment γ 305-355/356 plays a crucial role in calcium binding.

Dang et al (1985) used terbium fluorescence as a means to locate the fibrinogen calcium binding site on fragment D. It is

well established that the trivalent lanthanide, terbium, competitively inhibits the binding of calcium to the protein; binding of terbium to a calcium site results in the enhancement of terbium fluorescence by resonance energy transfer from tryptophan residues. By employing this elegant method of detection, the authors narrowed down the location of the D domain calcium site to lie between residues 311-336 of the γ-chain.

However, in their recent studies concerning the role of disulphide bonds near the calcium binding sites in fibrinogen, Procyk and Blomback (1987) reported that γ 326-339 is the important region of this chain for the binding of calcium.

Nieuwenhuizen et al (1979) originally found one high affinity site to have a different dissociation constant (k_d = 3.2 x 10^{-5}M) from the other two (k_d = 9 x 10^{-6}M). However, when the latter group of workers re-assessed their data in 1981, they concluded that the molecule had three identical binding sites (k_d = 1.9 x 10^{-5}M). This result is in contrast to that of Kemp et al (1983) who found one of the sites to have a higher affinity (k_d = 4.6 x 10^{-7}M) than the other two (k_d = 3 x 10^{-5}M).

The location of the third site is also contentious. Fibrinogen contains two fragment D entities; thus if there are three tightly bound calcium ions and the symmetry of the

fibrinogen molecule is taken into account, the remaining calcium ion must form a bridge between the two halves of the molecule. Marguerie (1977) observed that the initial rate of plasmin attack on fibrinogen is lower in the presence of calcium. According to the author, calcium influences the cleavage of the first one or two bonds involving the initial attack on the Aα-chains at the C-termini. This thus implicated the Aα-chains in calcium binding. This proposal, albeit tempting, was however disputed by two seperate sources.

Nieuwenhuizen and Gravesen (1981) showed that there was no difference in the number of calcium ions bound by fibrinogen and fragment X, thus precluding the involvement of the C-terminal parts of the Aα-chains in calcium ion binding. This view was supported by Ross and Kemp (1981) who found no evidence of calcium binding in their studies of calcium ion release during the early plasmin digestion of intact fibrinogen. Further studies by Nieuwenhuizen et al (1982b) supported the view that the C-terminal parts of the Aα-chains are not involved in calcium binding and suggested that the third high affinity site may be located in the central domain of the molecule. In fact later studies by workers from this laboratory (1983) concerned the purified fragments of fibrin(ogen) prepared by plasmin digestion or CNBr degradation. The authors found that E_3 (late fragment E) did not bind calcium, however a similar cyanogen bromide cleavage product did bind calcium, albeit with a lower

affinity ($K_d \simeq 10^{-4}M$) than that which these workers had previously observed in intact fibrinogen.

However Marguerie supported his earlier proposals by studies done by himself and Ardaillou in 1982. The authors found that molecules with intact Aα-chains possessed three high affinity binding sites whereas those fibrinogen molecules with partially degraded Aα-chains exhibited only two sites, and thus suggested that the integrity of the chain is required for interaction between the protein and cation. Thus two totally different proposals concerning the location of the third high affinity site exist. Some of these differences may be attributed to the lability of the C-terminal parts of the Aα-chains.

For calcium ions to play a regulatory role in fibrinogen function, the dissociation constant of binding sites on the molecule should be in the region of the physiological calcium concentration (ie. approximately $10^{-3}M$). The reported dissociation constants for the different types of affinity sites thus suggest that the high affinity sites ($K_d \simeq 10^{-6}M$) are an integral part of the structure of fibrinogen since their dissociation constant is significantly lower than the free Ca^{++} concentration in plasma (Hardy et al, 1983). However the low affinity sites ($K_d \simeq 10^{-3}M$) could play an important role in physiological phenomena. Little information is available regarding the latter sites apart from the fact that they are more numerous and less specific for calcium (Marguerie et

al, 1977). However since their dissociation constant is close to the physiological calcium concentration it is highly likely that calcium ions binding to these sites may have a regulatory function such as that concerned with fibrin polymerization.

The Shape of Fibrinogen

The overall structure of fibrinogen is still unknown and remains a controversial subject. Several conflicting models have been proposed ranging from a trinodular arrangement (Hall and Slayter, 1959) to a globular conformation (Koppel, 1966).

A comprehensive review of the main models put forward to date and the different methods used by various groups of workers for the determination of the latter, will be discussed in Chapter 4. However in order to put the voluminous amount of information into perspective, a summary of the techniques used and the principal basic structures proposed by various workers (in chronological order) can be seen overleaf in Table 1-(iv).

Table 1-(iv) : Proposed Models of Fibrinogen Conformation.

AUTHOR(S)	TECHNIQUE	SHAPE
Hall and Slayter (1959)	Electron microscopy (shadow casting)	Trinodular
Koppel (1966)	Electron microscopy (negative staining)	Globular (pentagonal dodecahedron)
Pouit et al (1972)	Electron microscopy (negative staining)	Globular (some flexibility)
Bachmann et al (1975)	Electron microscopy (freeze etching and shadow casting)	Cylindrical rod (some flexibility)
Hudry-Clergeon et al (1975)	Electron microscopy (negative staining)	Non-compact, flexible sphere
Marguerie et al (1975/76)	Neutron small angle scattering	Ellipsoid or 'banana' shape
Doolittle et al (1977)	Computer simulation	Trinodular
Mueller and Burchard (1978)	Light scattering	Flexible rod
Fowler and Erickson (1979)	Electron microscopy (shadow casting and negative staining)	Trinodular
Lederer (1979)	Small angle X-ray scattering	Flexible 'sausage'
Serralach et al (1979)	Data analysis	Flexible structure

[CONT/-]

AUTHOR(S)	TECHNIQUE	SHAPE
Estis and Haschemayer (1980)	Electron microscopy (negative staining)	Flexible rod
Hantgan (1981/82)	Steady state fluoresence polarization	Flexible structure
Mosesson et al (1981)	Scanning transmission electron microscopy (STEM)	Flexible trinodular
Weisel et al (1981/85)	Electron microscopy (rotary shadowing and X-ray crystallography)	Heptad model
Williams (1981)	Electron microscopy (rotary shadowing and negative staining)	Trinodular
Plow and Edgington (1982)	Immunochemical analysis	Flexible trinodular
Larsson et al (1987)	Dynamic laser light scattering (DLS)	Flexible structure

Part (2): Photosensitized Radioactive Labelling and Photosensitized Cross-Linking.

Photooxidation techniques

The use of photoaffinity labelling to explore the topography of proteins was originally proposed by Singh et al (1962). One approach involves the affinity labelling of ligand binding sites in the study of their structure-function relationship. This can be done by photoreactive probes incorporated into a compound normally binding to the site. Location of the amino-acid residues near or in the active site can thus be obtained by degrading the protein and identifying the labelled fragments. Examples of such sytems studied include the interaction of substrates and cofactors with enzymes, and hormones with their receptors (see review by Jori and Spikes, 1978).

However an alternative approach involves the use of photooxidation techniques. Photooxidations can be classified into two main groups, ie. direct oxidations in which the excited molecule is oxidised directly, and sensitized oxidations in which the initially excited molecule or 'sensitizer' indirectly promotes the oxidation of another molecule. Many processes induced by X-rays and γ-rays involve direct oxidations, however it is the sensitized mode of oxidation that is of relevance in the context of this work.

Dye-sensitized photooxidations can occur according to two different mechanisms (Meier, 1970), ie:

(a) Hydrogen abstraction (Type I), or
(b) Oxygen transfer (Type II).
(See Figure 1-6).

In type I the excited sensitizer interacts with another molecule directly, usually by transfer of a hydrogen atom or an electron. In the second class of reaction (type II) the sensitizer triplet (see Figure 1-7) reacts with oxygen, most commonly by energy transfer, to give an excited electronic state of oxygen, singlet oxygen, which reacts further with various acceptors in solution (Foote, 1968; Foote, 1976). The lifetime of the most important singlet oxygen species ($^1\Delta g$) is approximately 2 μseconds in neutral aqueous solutions and several orders of magnitude greater in non-polar solvents (Foote, 1976); it is characterized by an energy of 22kcal above the ground state. Dyes with reactive triplet states such as fluorescein usually react in the presence of air according to the oxygen-transfer mechanism.

Dye photosensitized oxidation has been used to modify certain amino-acids in proteins. (Ray, 1967; Westhead, 1972). This was done with the aim of identifying critical residues at, for example, the catalytic sites of enzymes which are involved in the enzyme's function. However modification of such amino-acids was difficult to detect and was achieved only by careful

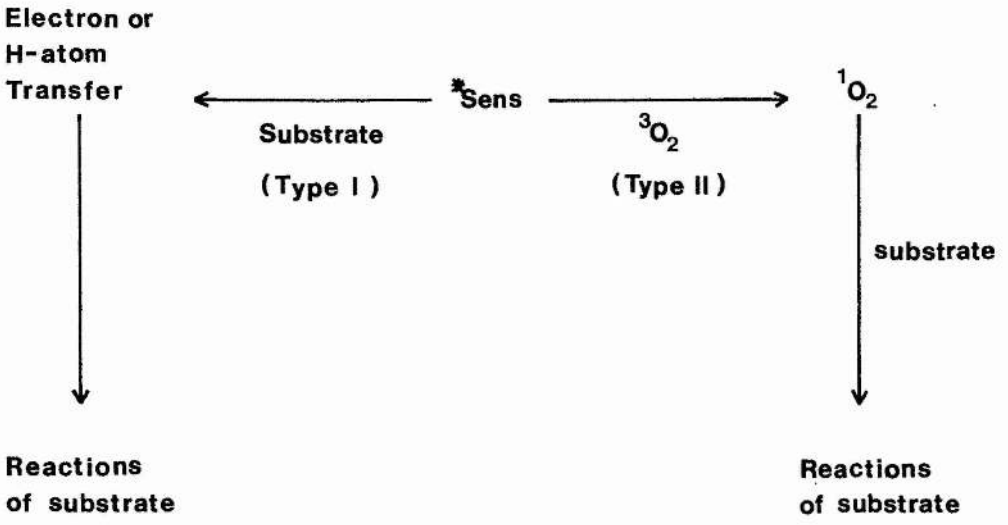


Figure 1-6: Type I and II sensitized photooxidation mechanisms.

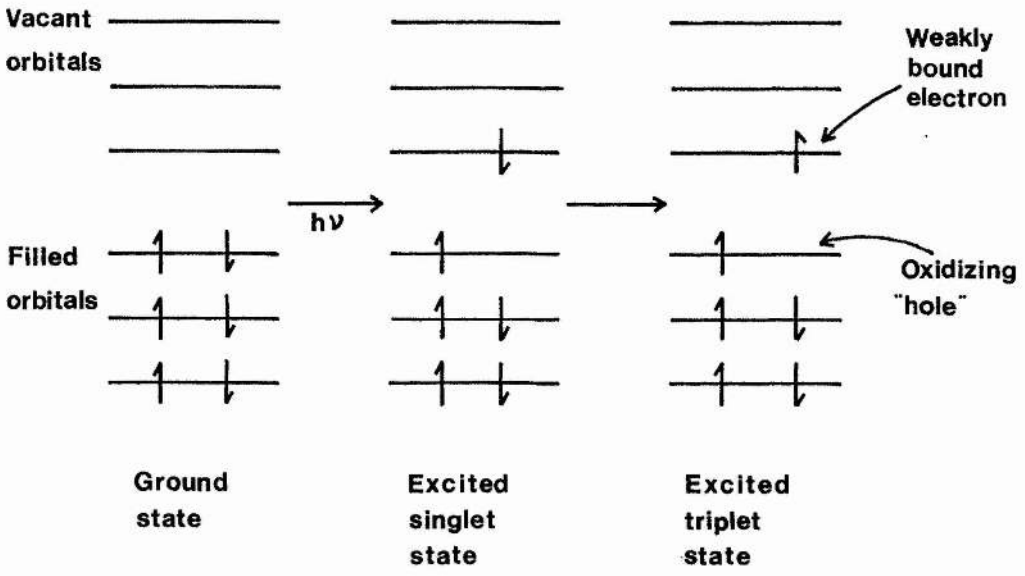


Figure 1-7: Electronic energy states.

selection of the dye and rigid reaction conditions; such experiments also involved the use of elaborately designed apparatus.

Brandt et al (1974) suggested that under certain experimental conditions the technique could be extended by using a dye or dye-ligand conjugate as a sensitizer with affinity for a protein binding site, thus favouring coupling to amino-acids in the binding site.

However an interesting proposal was put forward by Hemmendorff et al (1981) who developed a method for the covalent coupling of non-coloured low molecular weight substances to a protein upon excitation with visible light of a dye (fluorescein) in the presence of the protein and non-coloured substance. The coupling obtained was found to occur on solvent-exposed parts of proteins and was easily detectable since the low molecular weight label or tag used was tritiated tryptophan ([^{3}H]trp). This method of photosensitized radioactive surface labelling was selected for further development and assessment in these studies, and formed the basis of the rationale behind techniques used in this work.

Mechanisms

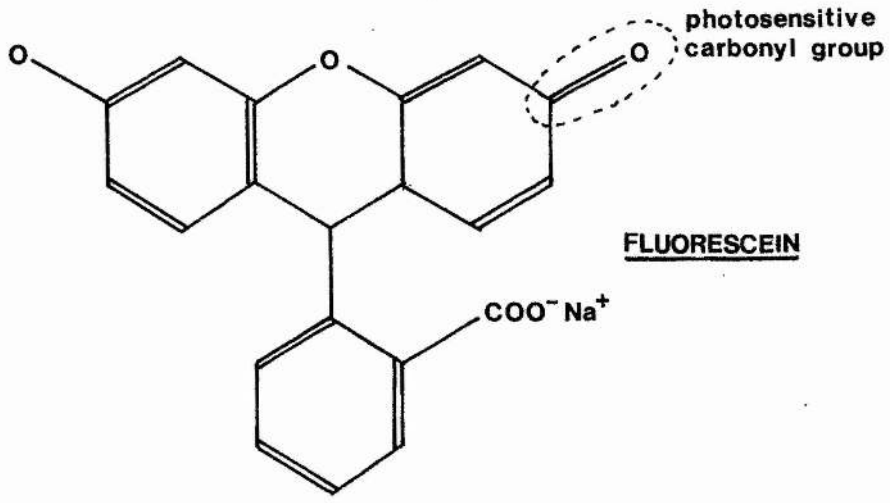
Fluorescein (molecular weight : 332) is a heterocyclic dye of the xanthene series which is insoluble in water, but soluble

in dilute alkali. Fluorescein and related dyes are known to be efficient photosensitizers and this activity has been shown to be due to the light-induced conversion of the dye to a reactive species which represents the dye in the triplet state (Lindqvist, 1963; Kasche and Lindqvist, 1964;). It is the carbonyl group in fluorescein which has been shown to be photosensitive (see Figure 1-8).. Fluorescein may also react when in the excited singlet state but evidence suggests that the triplet state is responsible for the photoactivity of the dye, due to the longer lifetime of this state (Lindqvist, 1963). The generation of fluorescein molecules in the triplet state is thus a key factor in the reaction.

The excited molecules can then transfer the energy of this state to other molecules, thereby starting specific reactions. If, as seen earlier, oxygen is present, triplet fluorescein reacts with oxygen and this results in the generation of the reactive species, singlet oxygen. The latter then continues the energy transfer process by photooxidising target molecules in solution.

(i) Photosensitized radioactive labelling

In the photosensitized radioactive labelling experiments carried out in this study, the reaction mixture was stirred using a magnetic stirrer, thus ensuring oxygen saturation and therefore the generation of the reactive singlet oxygen species. (For apparatus used, see Figure 2-6). The reaction solution



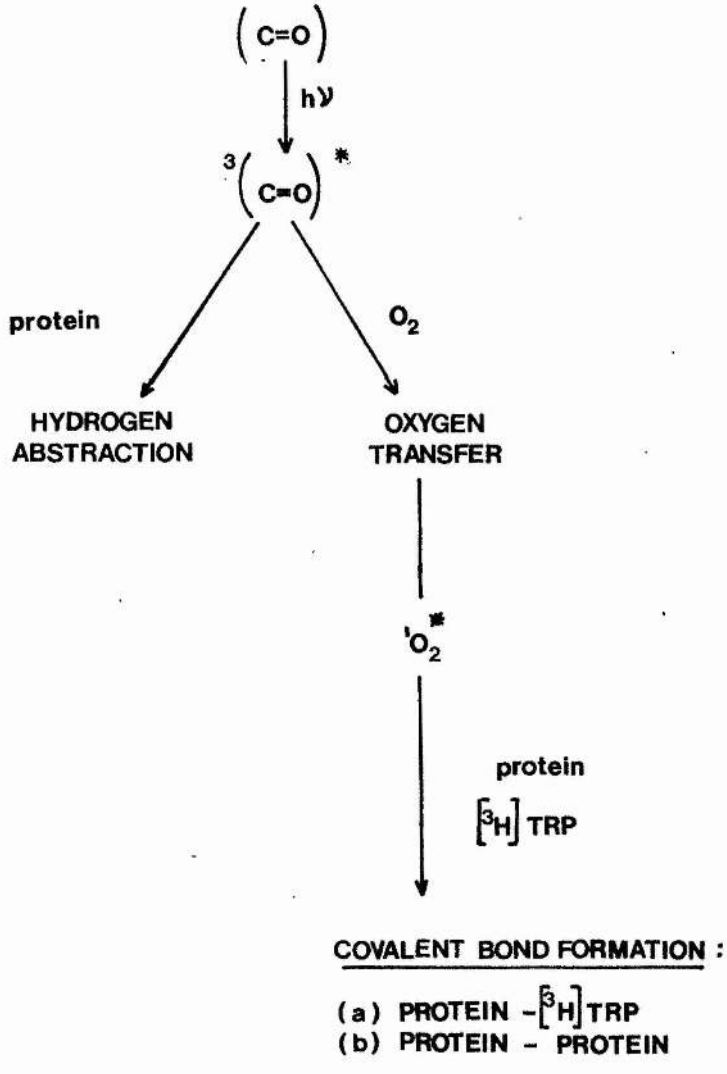


Figure 1-8: Dye-sensitized radioactive photolabelling and cross-linking.

consisted of a mixture of protein, fluorescein, and radioactive tryptophan (see Figure 1-8). Singlet oxygen thus transfers its energy both to amino-acid residues on the polypeptide chains of the protein as well as to free ligands in solution, in this case [^{3}H]trp. The latter is thus converted to a free radical and reacts non-specifically with the protein surface. Covalent bonds are formed and the amount and position of binding of [^{3}H]trp can be investigated. This information will indicate which parts of the molecule are exposed to solvent and are therefore surface-orientated.

Covalent bonds formed, however, may be of two types (see Figure 1-8) namely (a), which is essentially the labelling reaction and (b), which consists of the protein cross-linking reaction. In the case of radioactive photolabelling reactions the latter is an unwanted side-reaction and conditions must be optimized so that (a) may be the predominant reaction occurring (see Chapter 3, Section 2). The degree of cross-linking is easily monitored by examining the irradiated protein on SDS-polyacrylamide gels.

(ii) Photosensitized cross-linking

Although the formation of stable protein cross-links is considered to be an unwanted side reaction in radioactive photolabelling, the potential of this reaction was realized and was developed as an independent means of investigation.

The technique follows similar chemistry to that of photosensitized radioactive labelling with one major difference, - no free [^{3}H]trp is added to the system. Consequently the singlet oxygen acts as a cross-linking reagent, transferring its energy to protein molecules which form cross-links with any other protein (intermolecular cross-linking) or parts of the same protein (intramolecular cross-linking) which are in close proximity. Intermolecular cross-linking is favoured by higher protein concentrations and thus the resulting ratio of inter : intramolecular cross-linking can be controlled.

Because of the minute size of singlet oxygen, the possibility of reported reagent-induced conformational changes which result using classical cross-linking agents such as glutaraldehyde (Mossesson et al, 1981) and carbodiimide (Doolittle, 1984) are eliminated. Singlet oxygen also has additional advantages, - cross-links formed are not dependent on rigid spacing and specific target groups for the establishment of successful cross-links. The resulting cross-links are very short in length and thus cross-linked species must originally have been in very close proximity. This is not the case using chemical cross-linkers such as the bifunctional reagent dimethyl suberimidate which reacts specifically with lysine residues (Kennedy et al, 1976) to introduce cross-links 0.97nm is length, and tetranitromethane which reacts exclusively with tyrosine residues (Williams and Lowe, 1971). As well as this, in contrast with the vast majority of other cross-linking procedures, the

reaction is not directed at lysine residues and therefore it is possible to carry out plasmin digestion on the cross-linked material and gain additional information regarding the topography of the protein in this way.

A similar type of experiment was carried out by Girotti et al (1979) who showed that extensive inter-subunit cross-linking resulted when a mixture of haemoglobin and methylene blue was exposed to light. The authors demonstrated that the rate of cross-linking was enhanced by D_2O, indicating that singlet oxygen does indeed play a key role in the photooxidation reaction.

Part (3) : Aims of This Study

Despite all the research carried out on fibrinogen, certain basic aspects regarding, for example, the overall conformation of the molecule, remain unresolved. To date no single model proposed has been met with universal acceptance. In addition, the importance of calcium in influencing the structure and physiological properties of fibrinogen cannot be underestimated and remains the subject of much study and debate.

In this study two techniques have been selected for development and assessment, namely photosensitized radioactive surface labelling and photosensitized cross-linking. The main aim of this work was to develop the latter methods with a view to examining the conformation of fibrinogen (and its derivatives) in its native state and under different solvent conditions, with particular reference to the influence of calcium. The effects of other ions, such as magnesium and sodium, on the conformation of the protein were also investigated.

CHAPTER TWO

MATERIALS AND METHODS

Section (A) : PREPARATIVE TECHNIQUES

1. The Purification of Fibrinogen

Materials

- plasma (donated by the Blood Transfusion Centre, Ninewell's Hospital, Dundee)

- lysine-Sepharose column (1 x 20cm) equlibrated in 0.05M Tris-HCl (Sigma), pH 7.5

- aluminium hydroxide gel (prepared by the method of Ikemori et al, 1975)

- saturated ammonium sulphate (BDH Analar)

- 23% saturated ammonium sulphate

- 'peak I' buffer, ie. 0.05M Tris-HCl, pH 8.6, containing 0.05M NaCl and 0.5mM $CaCl_2$

- DEAE-cellulose (Whatman DE52) column (2 x 25cm) equilibrated in peak I buffer

Method

Fibrinogen was purified from fresh frozen plasma and the method used was based on that of Lawrie et al, (1979) (see Figure 2-1).

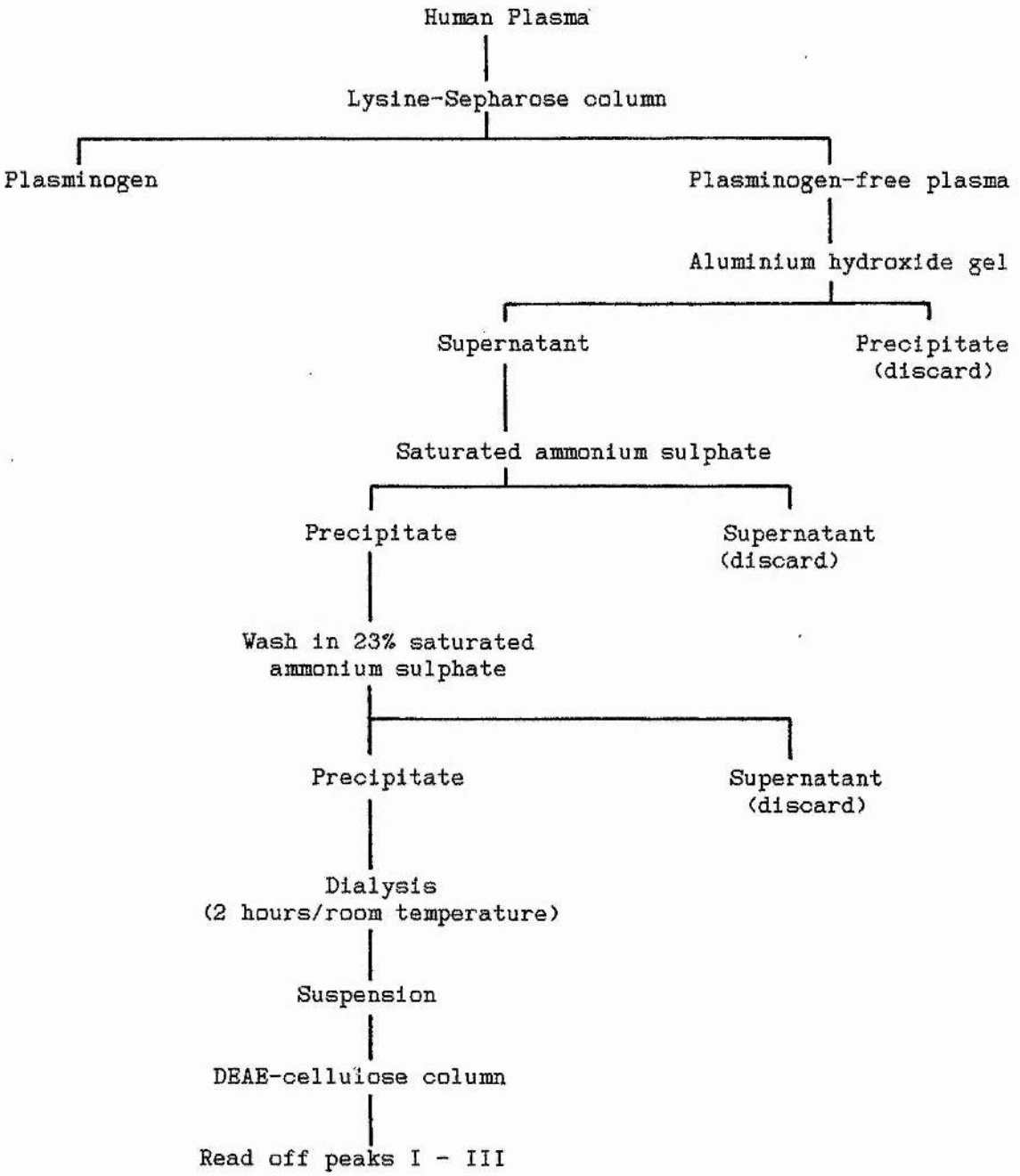


Figure 2-1: The Purification of Fibrinogen.

The plasma was thawed in a constant temperature water bath at 37°C and any precipitate was removed by centrifugation (2,500 rpm for 10 minutes). Plasminogen was removed from the plasma by passing the latter through a pre-equilibrated lysine-Sepharose column, as described by Deutsch and Mertz (1970). The eluate (ie. plasminogen-free plasma) was then brought to 37°C, a 1/10 volume of aluminium hydroxide was added, and the mixture was stirred for 15 minutes. The aluminium hydroxide was subsequently removed by centrifugation (3,000 rpm for 10 minutes) and the resulting supernatant retained. The precipitate was then washed with a volume of pH 7.5 buffer equal to half the original plasma volume, and the centrifugation procedure was repeated. The resulting supernatant was added to that from the first spin and the aluminium hydroxide precipitate was discarded.

A 1/3 volume of saturated ammonium sulphate was added slowly to the combined supernatants and stirred for 10 minutes at room temperature. The mixture was centrifuged (3,000 rpm for 10 minutes) and the supernatant discarded. After leaving the tubes to drain (upside down) for 10 minutes, the precipitate was washed with a volume of 23% saturated ammonium sulphate equal to one half the original volume of the combined supernatants, stirred for 10 minutes, and then centrifuged and drained as before.

The final precipitate was then resuspended in peak I buffer and dialysed against the said buffer (with buffer changes every

20 minutes) for two hours at room temperature. The contents of the dialysis sac were subsequently brought to 37°C and any resulting precipitate was spun down using a bench centrifuge, prior to the sample being applied to the pre-equilibrated DEAE-cellulose column. It should be noted that the DEAE-cellulose was precycled prior to use, according to the manufacturer's instructions.

The first protein peak (I) was eluted from the column with the peak I buffer. Two further peaks (II and III) were eluted with the same buffer first made 0.1M and then 1M in NaCl (see Table 2-i).

2. The Purification of Plasminogen

Materials

- plasma
- lysine-Sepharose column (1 x 20cm) equilibrated in 0.05M Tris-HCl, pH 7.5
- 0.05M Tris-HCl, pH 7.5, containing 0.3M NaCl
- 0.1M EACA, pH 7.5
- 0.2M EACA, 1M NaCl, pH 7.5
- ammonium sulphate
- 0.05M ammonium bicarbonate

Table 2-(i): Peak Analysis in the Purification of Fibrinogen.

BUFFER pH 8.6	ABSORBANCE (280nm)	FRACTION
0.05M Tris-HCl, 0.5mM $CaCl_2$, 0.05M NaCl.	≥0.1	Peak I fibrinogen
0.05M Tris-HCl, 0.5mM $CaCl_2$, 0.1M NaCl.	≥0.1	Peak II fibrinogen
0.05M Tris-HCl, 0.5mM $CaCl_2$, 1M NaCl.	Any third peak	Fibronectin etc; (discard)

- Sephadex G-25 (Pharmacia) column (1.5 x 25cm) equilibrated in 0.05M ammonium bicarbonate

Method

The first stage of this purification can be incorporated into that of fibrinogen (see Figure 2-1). The method used was based on those of Deutsch and Mertz (1970) and Rickli and Cuendet (1971).

Plasminogen was absorbed onto a pre-equilibrated lysine-Sepharose column and the resulting plasminogen-free plasma was used for the purification of fibrinogen as seen earlier. The column was then washed overnight with the Tris/NaCl buffer, pH 7.5. This procedure was followed by the elution of various fractions off the column in EACA buffer, pH 7.5 (see Figure 2-2). The EACA removes any plasminogen from the column by competing with lysine residues covalently bound to the Sepharose, to which the protein attaches. (The column may subsequently be cleaned after this stage with EACA/NaCl buffer, pH 7.5).

From the fractions collected, those with an A_{280} greater than 0.2 were pooled and 3.1g ammonium sulphate was added per 10ml of pool. The mixture was stirred to dissolve the salt and then left at 4°C overnight. Any resulting precipitate was spun down using a bench centrifuge, and then dissolved in approximately 1ml ammonium bicarbonate. The sample was then

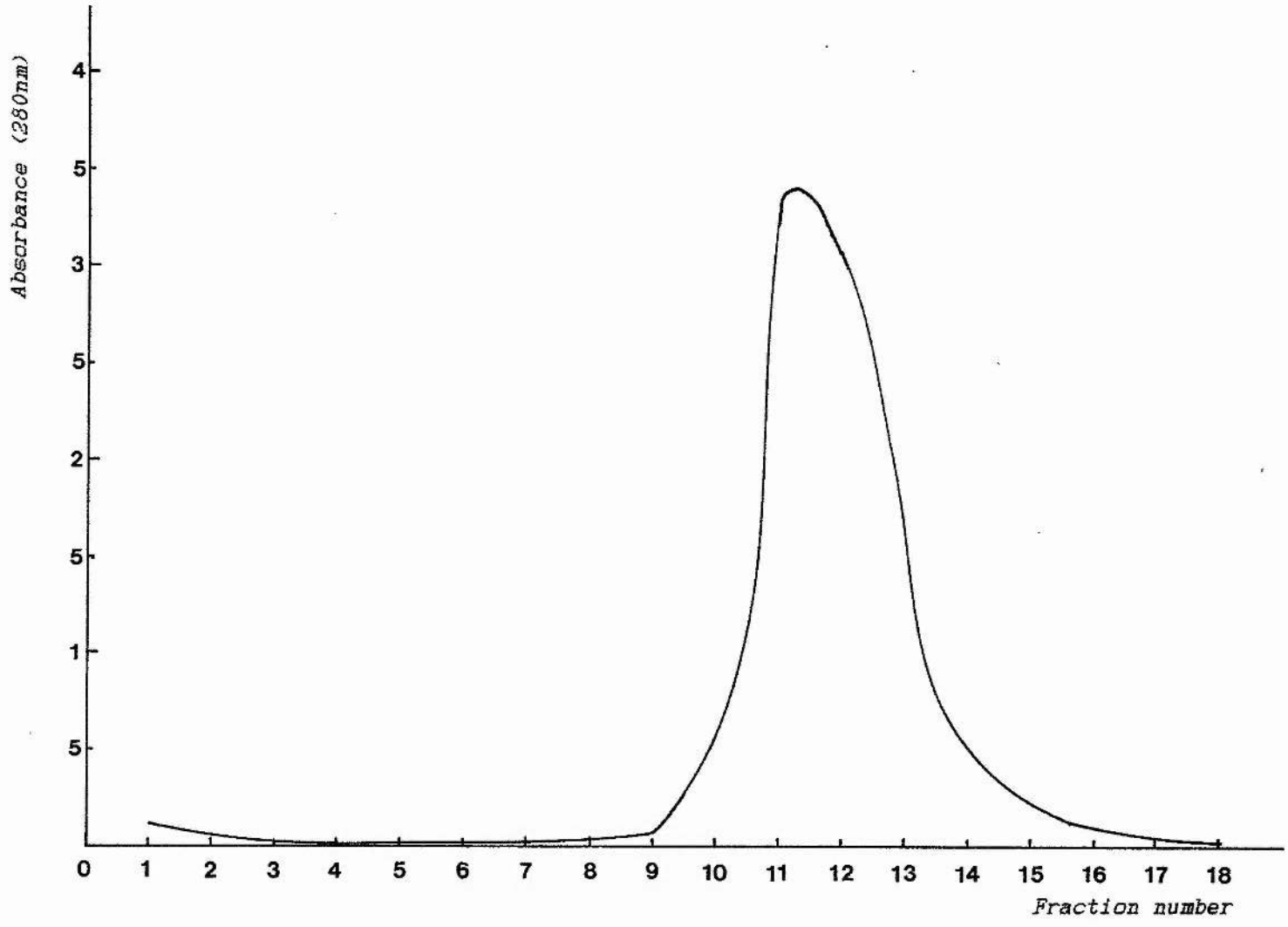


Figure 2-2: Elution profile for the first stage of plasminogen purification on lysine-Sepharose.

Elution buffer: 0.1M EACA, pH 7.5.

applied to a pre-equilibrated Sephadex G-25 column and of the fractions collected those with an A_{280} greater than 0.1 were pooled (see Figure 2-3). This pool was then divided into small aliquots (1ml) which were lyophilized and stored at -4°C until required.

3. Plasminogen Activation

Materials

- 'plasminogen buffer', ie. 0.05M Tris-HCl, pH 7.4, containing 0.012M NaCl

- streptokinase (Sigma) : stock solution of 2,000 Units/ml

Method

When required for the purpose of fibrinogen digestion, lyophilized plasminogen was resuspended in the above buffer. Streptokinase was then added to a final concentration of 10% (v/v) and the mixture was incubated at 37°C for 15 minutes.

4. Plasminogen and Plasmin Assays

(i) Plasminogen

Materials

As in 'Plasminogen Activation', plus :-

- S-2251 (Kabi Diagnostica); stock solution : 1.7mg/ml

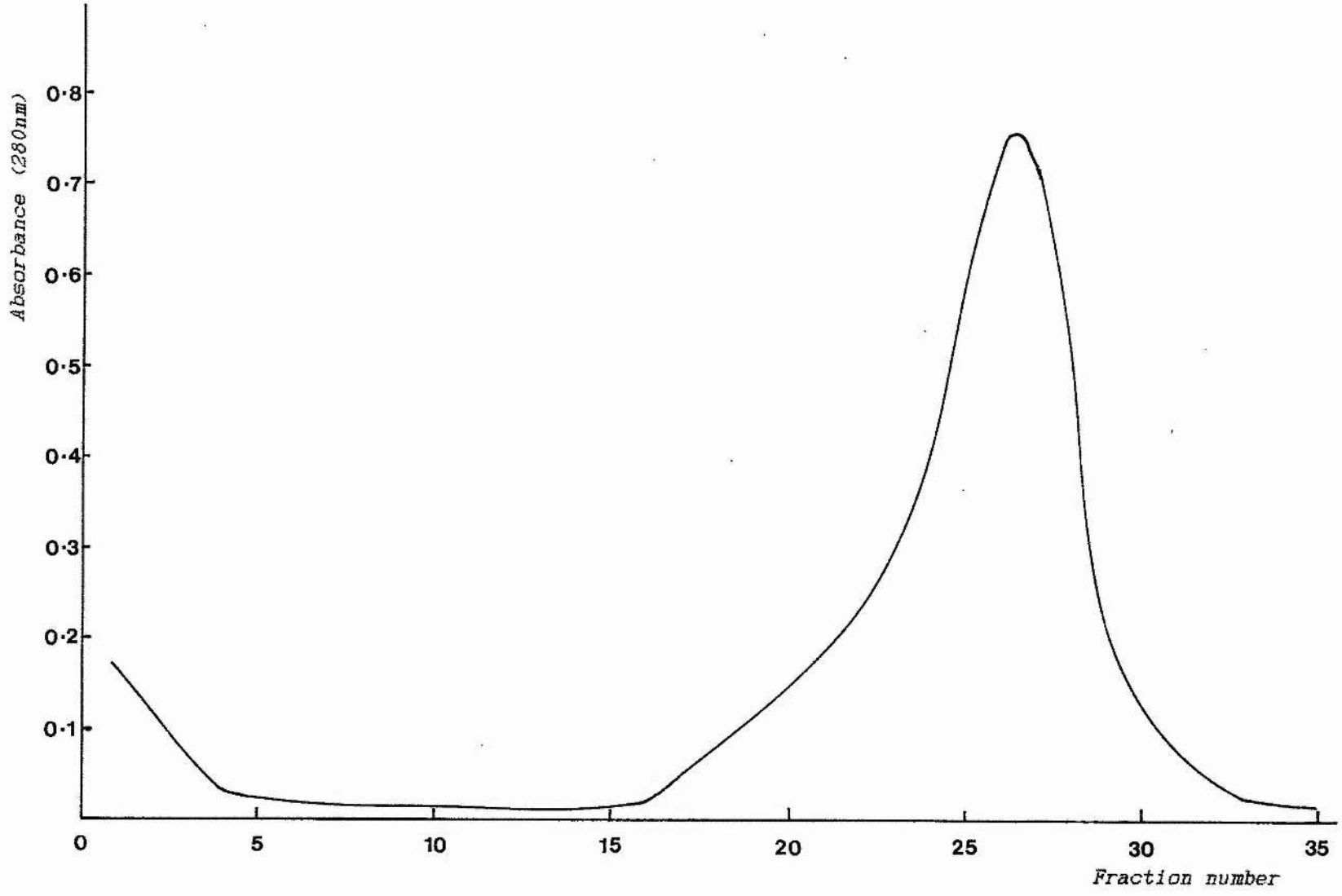


Figure 2-3: Elution profile for the final stage of plasminogen purification on Sephadex G-25.

Elution buffer: 0.05M Ammonium bicarbonate.

Method

100µl of streptokinase were mixed with an equal volume of plasminogen sample (resuspended in plasminogen buffer) in a cuvette. The mixture was then incubated at 37°C, in the heated cell carriage of a Cecil spectrophotometer, for 10 minutes. 700µl of plasminogen buffer (equilibrated at 37°C) were then added to the above, together with 100µl of the chromogenic substrate, S-2251. The change in absorbance at 405nm was monitored using a chart recorder.

The enzymic activity was then calculated and expressed in arbitrary units of Δ Absorbance/minute/mg of protein, where one such unit = Δ A_{405} of 0.01/minute; this is approximately equivalent to 0.002 C.I., or Caseinolytic Units.

(ii) Plasmin

Materials

- 'plasmin buffer' ie. 0.05M Tris-HCl, pH 7.4, containing 0.112M NaCl

- S-2251; Stock solution: 1.7 mg/ml

Method

800µl of plasmin buffer were added to 100µl resuspended enzyme sample, all at 37°C. 100µl of S-2251 were then added to the mixture. The change in absorbance at 405nm was recorded, and the enzymic activity calculated as previously described.

(iii) Examples

In a typical experiment, two plasminogen samples, A and B, resulting from different purifications, were assayed. S-2251 is a chromogenic substrate for both streptokinase-activated plasminogen (ie. plasminogen-SK) and plasmin. The principle of the reaction is as follows:-

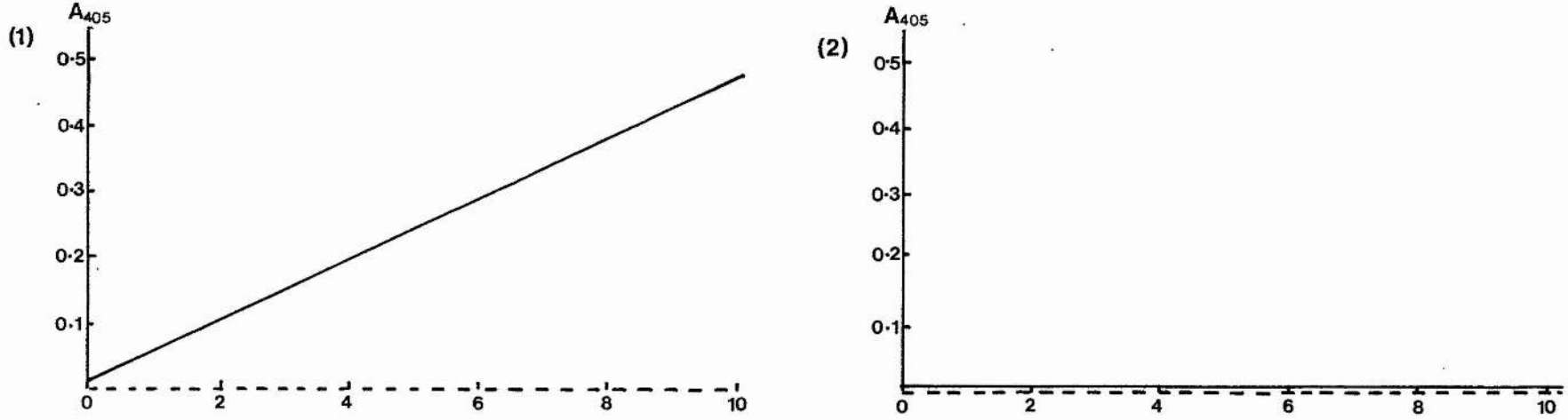
S-2251 ie:-

H-D-Val-Leu-Lys-pNA ----------enzyme----------> H-D-Val-Leu-Lys-OH + pNA

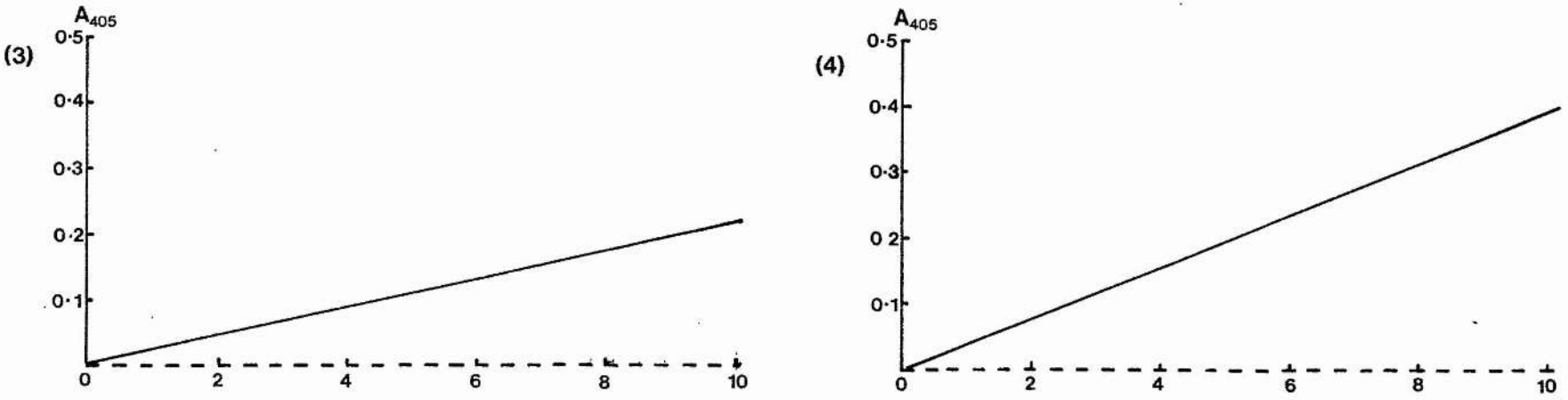
The method for the determination of activity is based on the difference in absorbance between the pNA (p-nitroaniline) formed and the original substrate. The rate of pNA formation, ie. the increase in absorbance at 405nm, is proportional to the enzymic activity and was determined using a spectrophotometer.

As can be seen from Figure 2-4, plasminogen A was found to be plasmin-free and therefore usable since this particular preparation will not tend to lose its activity by autolysis during storage. However, plasminogen B showed positive plasmin activity and was therefore discarded.

It should be noted that activity assays were routinely carried out on plasminogen preparations and rarely showed any plasmin activity. In fact, different plasminogen preparations showed very similar activities (ie. approximately 1,000



(i) Sample (A)



(ii) Sample (B)

Figure 2-4: Plasminogen (1,3) and plasmin (2,4) activity assays.

'arbitrary' units) which were always sufficient to digest fibrinogen to its core fragments D and E.

5(i) Preparation of Fragments D and E

Materials

- fibrinogen (peak I or II)
- plasminogen
- streptokinase (stock solution: 2,000 Units/ml)
- DEAE-cellulose column (2 x 25cm) equilibrated in 0.05M Tris-HCl, pH 7.5, containing 1mM $CaCl_2$

Method

The fibrinogen was dialysed overnight at 4°C, against five changes of the above pH 7.5 buffer. Plasminogen was then activated as described earlier and added to the dialyzed fibrinogen for digestion, using the relative quantities outlined by Lawrie et al (1977). The mixture was then incubated at 37°C for 4-6 hours and left at room temperature overnight. After the incubation period a digest sample was removed and run on an SDS-polyacrylamide gel (5% acrylamide) to check whether full digestion to the core fragments D and E had occured.

The digest mixture was then applied to the DEAE-cellulose column. After elution of one protein peak (ie. $A_{280} \geq 0.1$) the

remaining protein was eluted using a NaCl gradient of 0 to 0.5M NaCl.

5(ii) The Digestion of Photolabelled or Cross-linked Fibrinogen

The full digestion of photolabelled or cross-linked fibrinogen was carried out in a similar manner to that previously described. However prior to digestion the samples were dialysed into 0.05M Tris-HCl, pH 7.5, containing 0.1M NaCl and 1mM $CaCl_2$.

In the case of cross-linked samples the dialysis time was three hours, at room temperature, with buffer changes every 20 minutes. This was done so as to remove any fluorescein present and also to prepare the samples in the appropriate buffer for digestion.

With respect to the photolabelled fibrinogen samples, the dialysis time was 48 hours, with approximately eight buffer changes. This extended dialysis time was required since not only was there need to remove any fluorescein present, but also the excess radioactive [^{3}H]tryptophan label.

It should be noted that in the case of both cross-linked and photolabelled fibrinogen samples, separation of the core fragments simply by SDS-polyacrylamide gel electrophoresis (5% acrylamide) was adequate for the purposes of these experiments

and separation by ion exchange chromotography on DEAE-cellulose was therefore not necessary.

6. The Purification of Fluorescein

Fluorescein (Fisons Scientific) was further purified by precipitation from a 0.01M NaOH solution by the addition of glacial acetic acid as described by Andersson et al (1971). The procedure was repeated three times, after which the molar absorption coefficient of the purified fluorescein in 0.01M NaOH was 8.4×10^4 $M^{-1}cm^{-1}$ at 491nm. A 3mM solution of fluorescein was prepared and stored in darkness at 4°C until required.

Section (B) : ANALYTICAL TECHNIQUES

1. Polyacrylamide Gel Electrophoresis

Materials

- Gel Buffer : 0.1M Tris-HCl, 6M urea (BDH), 0.2% (w/v) SDS (BDH), pH 7.4 (adjusted with HCl).

- Chamber buffer : 0.1M Tris-HCl, 0.2% (w/v) SDS, pH 7.4

- Acrylamide (BDH)/Bisacrylamide (BDH) stock solutions:

  (i) 10% : 9.7% (w/v) acrylamide
  0.3% (w/v) bisacrylamide
  200ml gel buffer

  (ii) 5% : 4.7% (w/v) acrylamide
  0.3% (w/v) bisacrylamide
  200ml gel buffer

- Ammonium persulphate (BDH) solutions:

  150 mg/ml for the preparation of 3% gels
  120 mg/ml for 5% gels
  75 mg/ml for 10% gels

- TEMED (Sigma)

- Non-reducing medium : .8M urea
  3% (w/v) SDS

- Reducing medium : 8M urea
  3% (w/v) SDS
  3% (v/v) β-mercaptoethanol (BDH)

- Bromophenol blue solution (0.05% w/v)

- Glycerol

- Indian ink

- Stain : 3.3g Brilliant Blue R (Sigma: 75% purity)
  454 ml methanol
  454 ml distilled water
  72 ml glacial acetic acid

- Destain : 250 ml methanol
  675 ml distilled water
  75 ml glacial acetic acid

Method

In this work the procedure of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was used to determine the molecular weight of proteins (Weber and Osborn, 1969), assess the purity of protein preparations, investigate the degree of enzymic degradation, and quantitate cross-linking reactions. The relevant protein bands on polyacrylamide gels were also cut out and solubilized in photolabelling experiments for estimation of radioactive incorporation.

3% polyacrylamide gels were also used in these studies and the necessary acrylamide/bisacrylamide solution was prepared by dilution of the 10% stock solution. For preparation of the gels, 19ml of the required acrylamide/bisacrylamide solution were mixed with the TEMED solution and the appropriate ammonium persulphate solution (freshly prepared), to the final concentrations given in Table 2-(ii). The latter was then mixed thoroughly and quickly pipetted into glass gel tubes (approximate dimensions : 9.7 x 0.75 cm) which had been previously sealed at one end with parafilm. A gap of 1-1.5cm was left at the top of each tube and a few drops of distilled water were layered on top of each gel to prevent the formation of a meniscus. The gels were then left to set, a process taking approximately an hour, after which the distilled water was replaced by gel buffer. The gels were stored overnight at 4°C prior to use.

Normally 7-10μg of protein was applied to each gel. Non-reduced samples were mixed with an equal volume of non-reducing medium before application to the gels. Samples for reduction were mixed with an equal volume of reducing medium and boiled for five minutes.

All samples were then each mixed with approximately 10μl of bromophenol blue tracker dye and one drop of glycerol, and the whole volume was applied to the gel. (The bromophenol blue acts as a marker for the material's progress through the gel, whilst

Table 2-(ii): Final Concentration of Components for SDS-Polyacrylamide Gel Polymerization.

ACRYLAMIDE/BISACRYLAMIDE	3%	5%	10%
Ammonium persulphate (w/v)	0.075	0.060	0.050
TEMED (v/v)	0.150	0.125	0.100

the glycerol increases the density of the sample, thus reducing diffusion of the latter into the surrounding chamber buffer which is later layered over the samples).

The gels were then placed in a Shandon disc gel electrophoresis tank, chamber buffer was poured into each chamber (ie. upper and lower), and a current of approximately 5mA per gel was applied. Electrophoresis was allowed to proceed until the marker dye was approximately 0.5-1cm from the bottom of the gel tube. The gels were then removed from the glass tubes by the injection of chamber buffer (by syringe) down the sides of the tube and the position of the marker dye on each gel was marked with indian ink.

The gels were then placed in stain solution for a minimum one hour period. After this procedure the gels were removed from the latter solution and placed in destain which was changed 3-4 times over a 24-hour period.

After sufficient destaining, the gels were scanned using a Vitatron TLD 100 densitometer and, where necessary, the mobility of the protein bands was calculated using the following formula :-

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{distance of tracker dye migration}}$$

For each electrophoretic run, gels loaded with a mixture of reduced, standard proteins were included. The standards used were:

(a) myoglobin (MW : 17,000),
(b) soya bean trypsin inhibitor (MW : 20,000),
(c) carbonic anhydrase (MW : 29,000),
(d) ovalbumin (MW : 45,000) and
(e) bovine serum albumin (MW : 116,000).
(All purchased from Sigma Chemical Co;).

By plotting the mobility of the standards against $\log_{10}$ of their known molecular weights, a calibration graph was obtained and from this the molecular weights of unknown proteins was estimated.

In cross-linking experiments the amount of cross-linking was quantified using scans of the gels obtained by 'Data Collect' and 'Data Anal' computer programmes. The Apple computer interface was directly linked to the Vitatron scanner 'record' output and the programmes used were adapted for compatibility with the densitometer by Dr. Graham Kemp.

2. Laemmli (Discontinuous) Gels

Materials

- Acrylamide/bisacrylamide stock solution:
  30% (w/v) acrylamide
  0.8% (w/v) bisacrylamide

- 1M Tris-HCl, pH 6.8

- 1M Tris-HCl, pH 8.8

- 0.2M EDTA-NaOH, pH 8.0

- 10% (w/v) SDS

- ammonium persulphate (10 mg/ml)

- TEMED

- Electrode buffer : 0.025M Tris-HCl,
  0.192M glycine,
  0.1% (w/v) SDS,
  pH 8.3.

- Stacking gel, layering buffer : 1M Tris-HCl,
  0.2M EDTA,
  10% (w/v) SDS,
  pH 6.8.

Method

The 'discontinuous' method of gel electrophoresis in acrylamide gels containing SDS was developed by Laemmli (1970).

The percentage acrylamide combination of stacking:separating gel ratios used in these studies was 3:7½% and these gels were made up according to Table 2-(iii).

The separating gels (7½%) were prepared first and left to set for an hour in glass tubes (9.5 x 0.75 cm), previously sealed with parafilm at one end. During this period the gels were layered with a few drops of distilled water. The stacking gel solution (3%) was then prepared and pipetted on top of each of the separating gels, leaving a space of 1-1.5cm at the top of the tube. These gels were also layered with distilled water whilst setting. The length ratio between stacking and separating gels was approximately 1:10, usual lengths being approximately 7.5cm for the separating gel and 0.75cm for the stacking gel. In order to facilitate pipetting, the glass tubes were previously marked on the outside (with a marker pen) at approximately 7.5cm and 8.3cm from the closed ends. After the stacking gels had set (approximately one hour) the distilled water layer was substituted with gel layering buffer and the gels were left overnight at 4°C prior to use.

Sample application onto Laemmli gels is the same as for SDS-PAGE and the Laemmli gels were also run according to the latter procedure. Differences between the two techniques lie in the fact that Laemmli gels are run at the reduced current of 3mA per gel and the buffer used in the Shandon tank chambers is of a

Table 2-(iii): Final Concentration of Components Required for the Polymerization of 3% Stacking Gels and 7½% Separating Gels.

SOLUTION	STACKING GEL	SEPARATING GEL
Acrylamide:bis	3%	7½%
Tris-HCl, pH 6.8	0.065M	---
Tris-HCl, pH 8.8	---	0.375M
EDTA, pH 8.0	0.002M	0.002M
10% (w/v) SDS	0.1%	0.1%
Ammonium persulphate	0.1%	0.05%
TEMED	0.1%	0.05%

different composition and pH to the chamber buffer previously used in the SDS-PAGE method.

3. Detection of Protein

The presence of protein in solution was usually determined by measuring the absorbance of solutions at 280nm. The $E_{1\%}$ (absorption coefficient) of the proteins used in this work (at 280nm) are known (Marder et al, 1969) and are shown in Table 2-(iv).

When the concentration of the samples was too low to determine the concentration of the protein accurately using the above method, protein estimations were carried out using the method of Bradford (1976). The latter method was also used in all cases where fluorescein had been added to the system. All measurements using the Bradford method were carried out in duplicate.

Table 2-(iv): $E_{1\%}$ Values (280nm) of Some Proteins Used in This Study.

PROTEIN	ABSORPTION COEFFICIENT ($(E_{1\%})$ at 280nm)
Fibrinogen	15.1
Fragment X	14.2
Fragment D	20.8
Fragment E	10.2
Plasminogen	17.0

Section (C): EXPERIMENTAL TECHNIQUES

1. Photosensitized Radioactive Labelling

The technique used was based on that of Hemmendorff et al (1981). An outline of the experimental protocol can be seen in Figure 2-5. However various problems were encountered at certain stages and a detailed account of how these were tackled and overcome can be seen in Chapter 3, Section 2 (Development and Optimization of Techniques).

The protein to be photolabelled was first dialysed into the appropriate buffer. 0.9ml of protein (0.3 - 0.6 mg/ml) were then mixed with 0.5ml 3mM fluorescein (purified as previously described) and 0.1ml of a 0.05mM radioactive tryptophan mix. (It should be noted that the latter represents a mixture of L-[G-^{3}H]-tryptophan (Amersham International, plc) diluted with 'cold' L-tryptophan (BDH) to give a constant specific activity of 3.374 μCi/nmole). The apparatus used in a typical irradiation experiment can be seen in Figure 2-6. The mixture was stirred (thus ensuring that the solution was oxygen-saturated) in a sample vial (diameter : 2cm) which was placed in an ice bath at a constant distance of 13.5cm from the light source. The reaction mixture was then irradiated for a set period of time with a 500 or 1,000 Watt Philips lamp. After irradiation, fluorescein and excess tryptophan were removed from the sample (see Chapter 3, Section 2) and labelled proteins were seperated

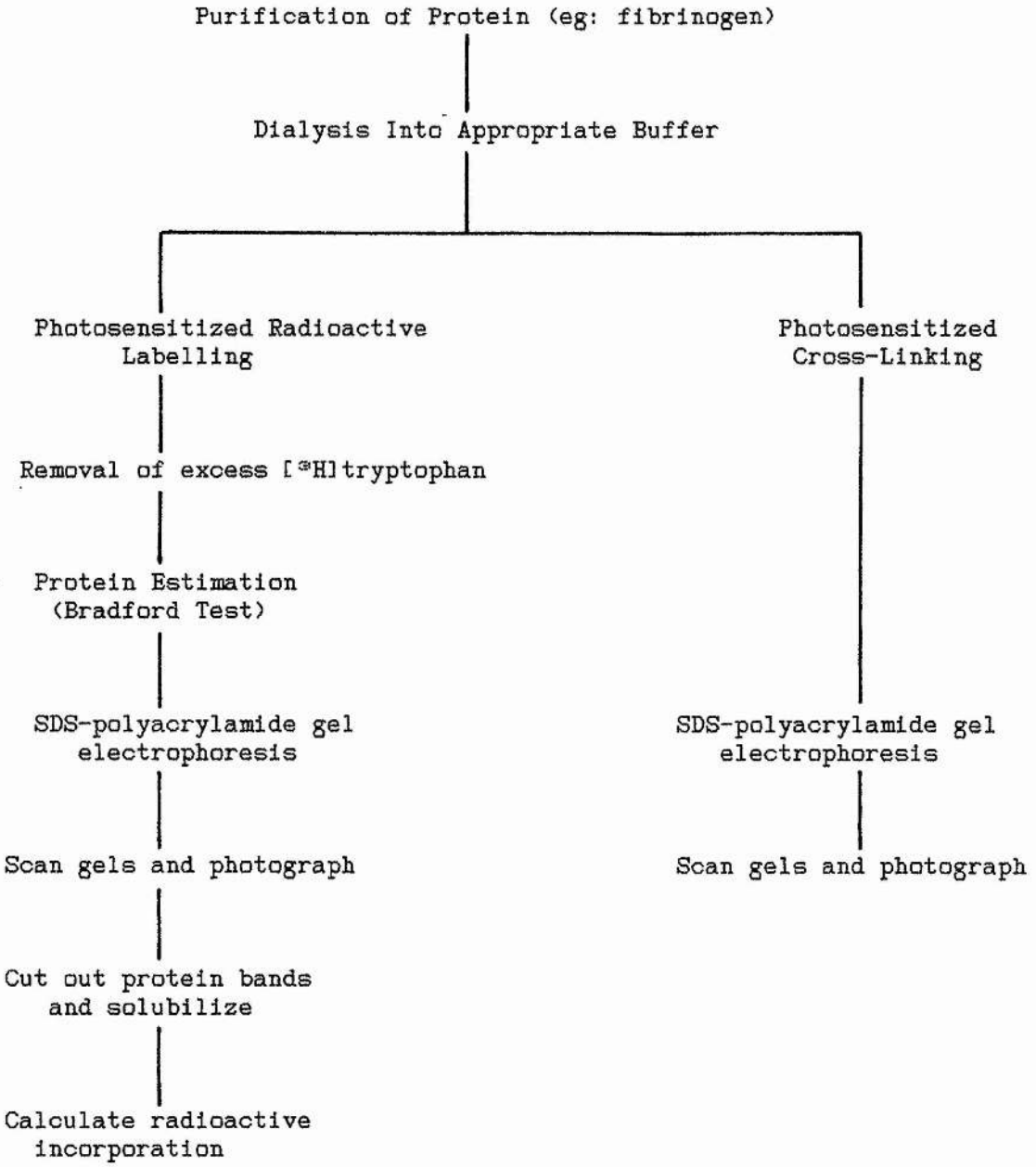


Figure 2-5 : Experimental Protocol.

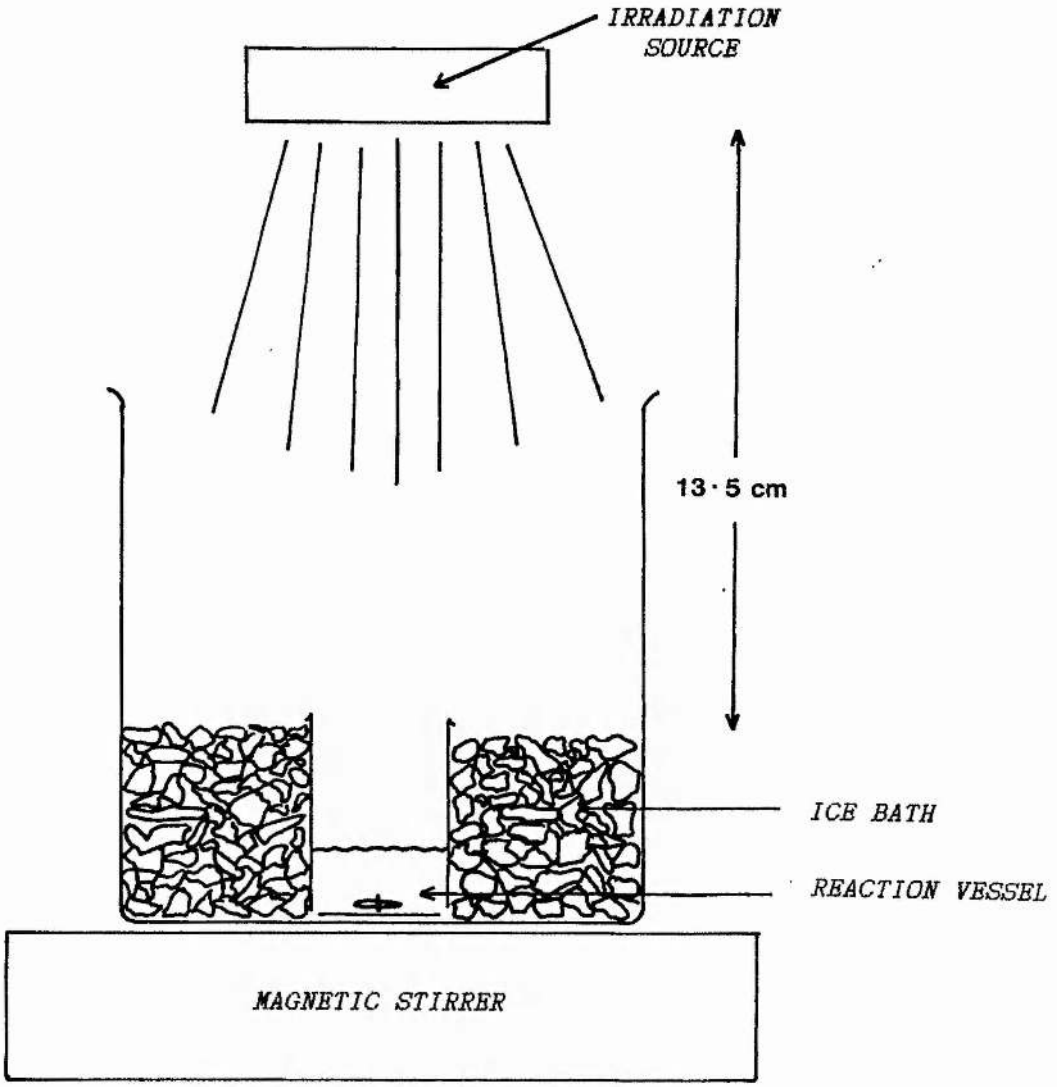


Figure 2-6: Apparatus used for sample irradiation.

by SDS-PAGE. Whenever necessary the gels were scanned using a Vitatron TLD 100 densitometer.

Stained bands were cut out of the gel using either a 'blade cutter' (1mm slices) or a scalpel. The gel slices were then solubilized in 0.5ml of 30% (w/w) hydrogen peroxide (BDH) in sealed vials at 75°C for eight hours, on a constant temperature heating block (Grant). After solubilization and cooling of the vials to room temperature, 4ml of toluene scintillant (prepared as described by Cooper, 1977) were added to each vial and the contents were mixed thoroughly using a vortex mixer. The amount of radioactivity in each vial was then measured in triplicate using a Packard Tri-Carb 300 CD scintillation counter.

In addition to the stained protein bands, the rest of the gel was also cut up and solubilized. The average of the radioactivity measurements from such portions of the gel were subtracted as background values from the radioactivity measurements of the protein-containing vials.

2. Photosensitized Cross-Linking

As with photosensitized radioactive labelling, a description of the optimum conditions for this technique is given in Chapter 3, Section 2; however a rough outline of the experimental protocol can be seen in Figure 2-5. The technique of fluorescein-activated cross-linking follows similar chemistry

and uses similar apparatus (Figure 2-6) to that of photosensitized radioactive labelling with one major difference, - no tryptophan is added to the system.

Thus 1ml of protein (0.7 mg/ml) in the appropriate buffer was mixed with 0.5ml of 3mM fluorescein. The irradiation procedure was carried out as previously described and cross-linked products were subsequently analysed by SDS-PAGE and/or Laemmli gels. The gels were then scanned using a Vitatron TLD 100 densitometer and the calculated peak areas were used to determine the relative amounts of protein present.

CHAPTER THREE

RESULTS

Section (1): Purifications

Fibrinogen

Fibrinogen was purified from human plasma by a method adapted from that of Lawrie et al (1979), as outlined in the previous chapter. The primary objective was to obtain a good yield of high quality fibrinogen, having as intact an Aα-chain as possible.

Figure 3-1 shows the elution profile of the DEAE-cellulose column used in the final stage of the purification. Peak I characteristically contains the most intact fibrinogen and peak II a somewhat less intact fibrinogen type. This difference can be seen in Figure 3-2 where discontinuous Laemmli gels (3:7½%), which have a higher band resolution than SDS-polyacrylamide gels, were run in order to highlight such differences. The Aα-chain band of reduced peak I fibrinogen is more intact than that of the peak II gel, in which the Aα-chain has been partially degraded. According to the work of Mosesson et al (1974) and Semeraro et al (1977), this is the result of the digestion of the labile C-terminal parts of the Aα-chains which gives rise to the heterogeneity of these chains.

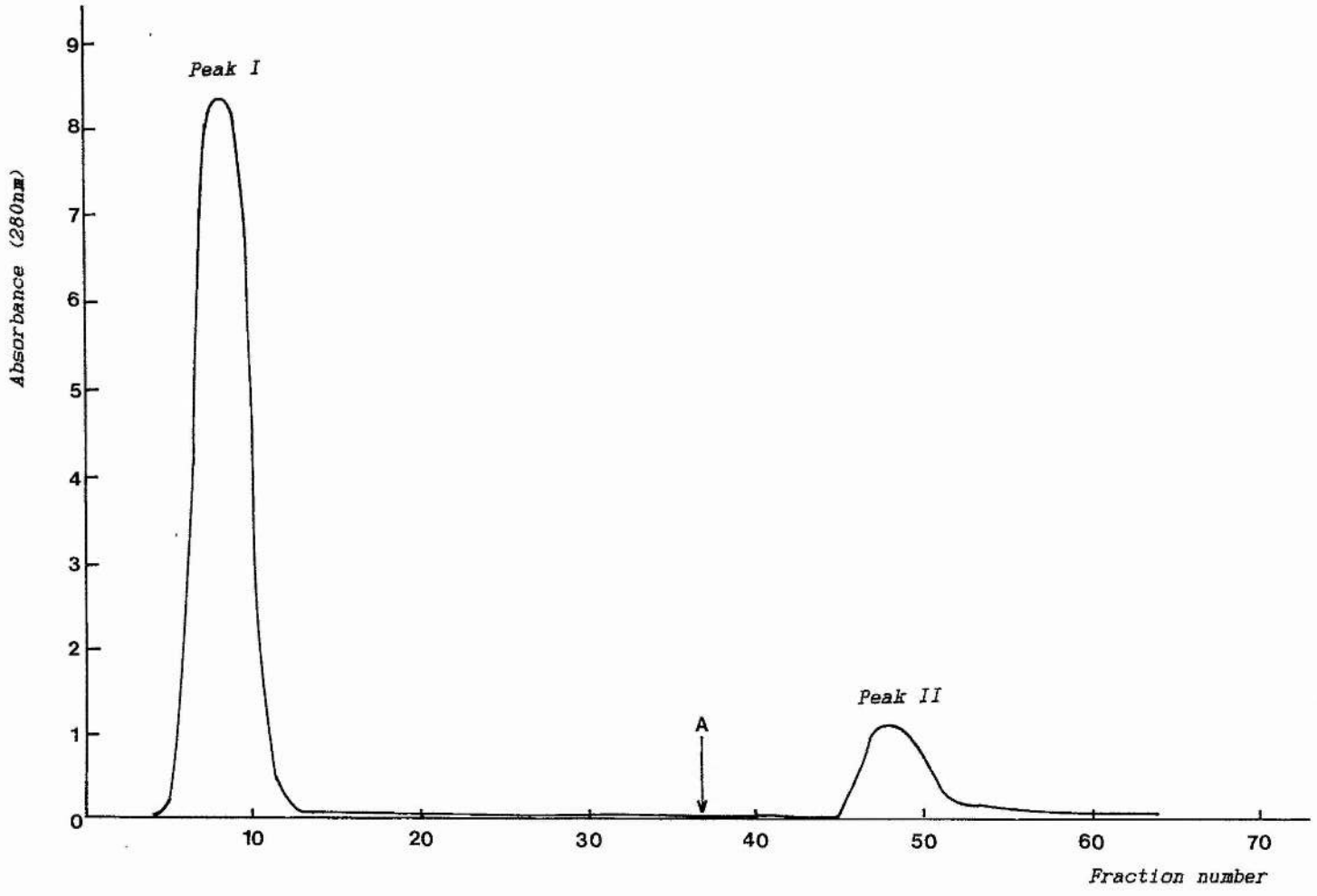


Figure 3-1: Elution profile for the purification of fibrinogen on DEAE-cellulose.

Elution buffer: 0.05M Tris-HCl, pH 7.5 containing 0.05M NaCl, 0.5mM $CaCl_2$.

A = [NaCl] raised to 0.1M.

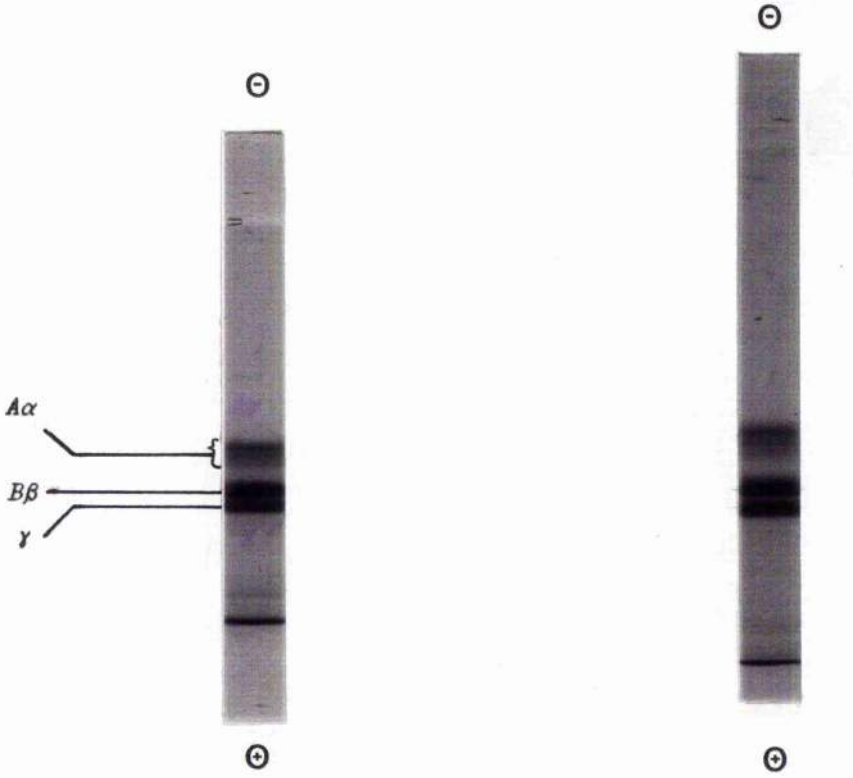


Figure 3-2: Purified fibrinogen after reduction. (Analysed on Laemmli, 3:7½ % gels).

The graph in Figure 3-1 represents a satisfactory preparation in which a high yield of good quality peak I fibrinogen resulted. Figure 3-3 shows the appearance of peak I fibrinogen, both reduced and non-reduced. on 5% and 3% SDS-polyacrylamide gels respectively. The decreased band resolution for the reduced 5% SDS gels as compared to the Laemmli gels (Figure 3-2) should be noted.

Fragments D and E

The digestion of fibrinogen by plasmin was carried out by the method described in the last chapter. This was done in the presence of calcium (1mM) since, as seen earlier, Haverkate and Timan (1977) showed that D(cate) (ie. the final fragment D product resulting when calcium is present) is protected from further plasmin attack by calcium ions. (N.B. From here on this fragment will simply be referred to as fragment D).

Figure 3-4 shows a 5% SDS-polyacrylamide gel of the digestion mixture, where the digestion resulted in the core fragments D and E. The mixture was then applied to a precycled DEAE-cellulose column, the elution profile of which can be seen in Figure 3-5. After elution of one protein peak in 0.05M Tris-HCl, pH7.5, containing 1mM $CaCl_2$, the remaining protein was eluted using a sodium chloride gradient of 0 to 0.5M NaCl.

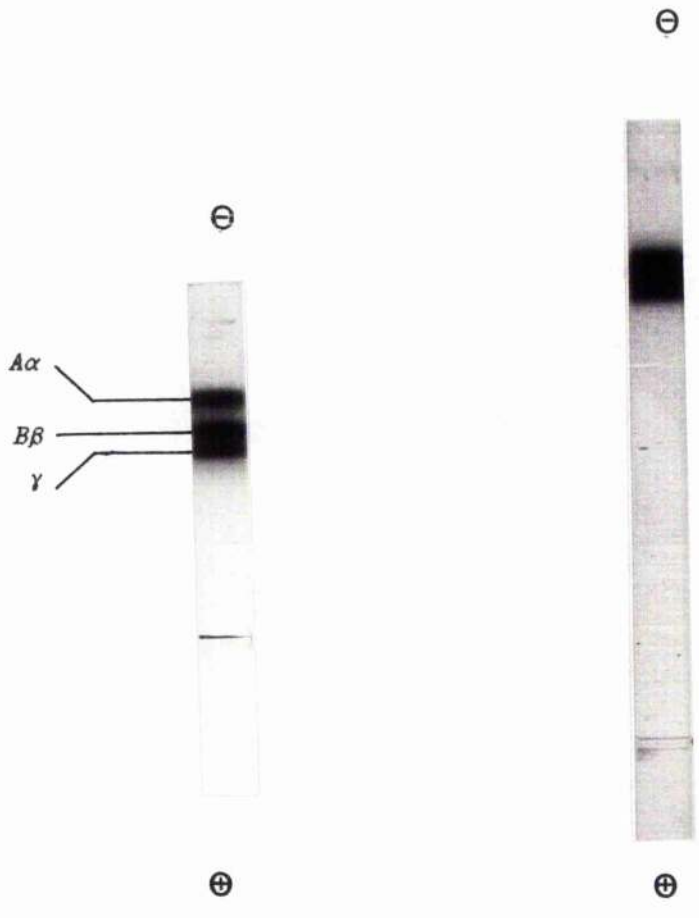


Figure 3-3: SDS-polyacrylamide gels of reduced (5% acrylamide) and non-reduced (3% acrylamide) fibrinogen.

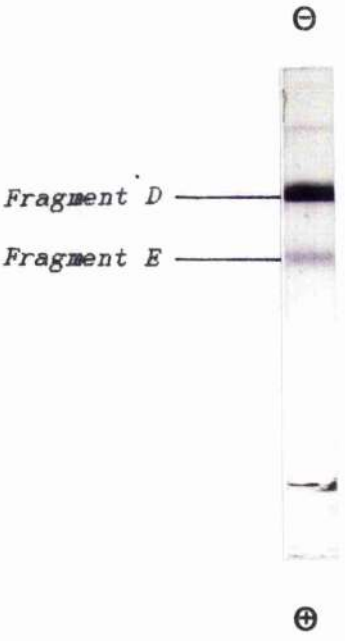


Figure 3-4: The digestion of fibrinogen to fragments D and E. (Photograph of 5% acrylamide gel).

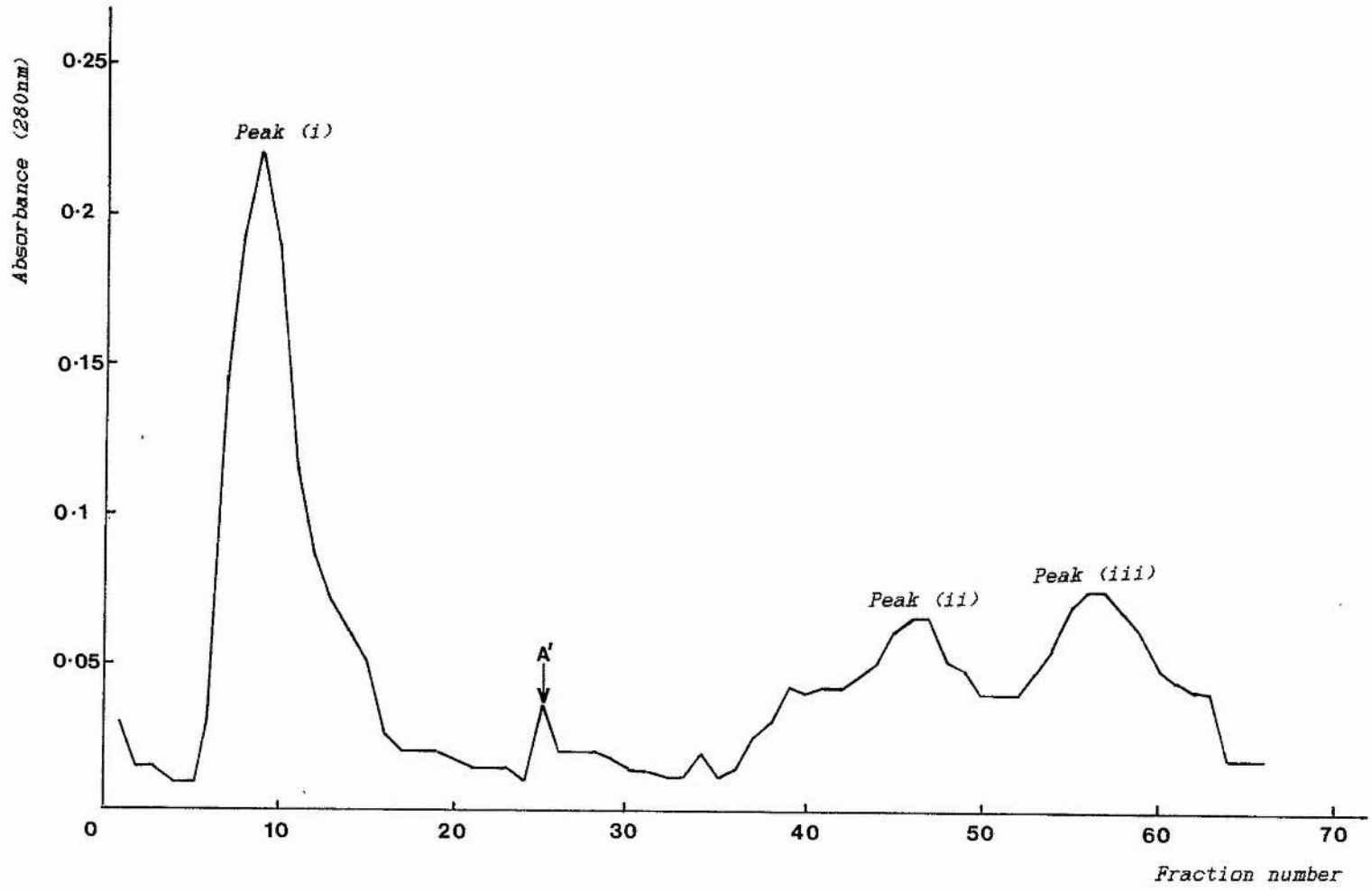


Figure 3-5: Elution profile for the seperation of fragments D and E on DEAE-cellulose.

Elution buffer: 0.05M Tris-HCl, pH 7.5, containing 1mM $CaCl_2$.

A' = NaCl (0 - 0.5M) gradient applied.

Peak (i) was found to consist of fragment D having an apparent molecular weight (calculated from SDS-polyacrylamide gels) of 87,000, whilst peak (iii) consisted of fragment E with an apparent molecular weight of 50,000. Gels of peak (ii) showed a mixture of the two protein species.

Section (2): Development and Optimization of Techniques.

As seen previously, the primary objective of this work was to develop two techniques, namely photosensitized radioactive labelling and photosensitized cross-linking, with the aim of applying these methods to investigate the topography of fibrinogen. In very simple terms, photosensitized labelling indicates how much of the molecule is surface-orientated whilst cross-linking indicates which parts of the molecule are closest together.

Both techniques have three main advantages in common, namely:

(i) All experiments are carried out in solution; thus fibrinogen is labelled or cross-linked in its native state and no rigorous conditions are required for these techniques.

(ii) The initiation and duration of the reaction can be easily controlled and,

(iii) both processes occur via highly reactive, short-lived intermediates.

Conditions for both techniques had to be optimized, hence a great deal of the initial work was concerned with experimental design.

A rough outline of the experimental protocol can be seen in Figure 2-5 (Chapter Two). Various problems were encountered at

the different stages and the following section deals with how the latter were tackled and overcome.

Light Source.

Initial photosensitized radioactive labelling experiments of fibrinogen, in 0.05M Tris-HCl, pH 7.5, containing 2mM $CaCl_2$, (from here on referred to as the Tris/$CaCl_2$ buffer) were carried out using a 500 Watt lamp (Philips) and the resulting degree of incorporation was found to be insufficient for subsequent analysis. However, when this was changed to a 1,000 Watt lamp (Philips Watastar 1000), the amount of radioactive incorporation increased by approximately 13 times. This result can be seen in Table 3-(i) where time of irradiation is 60 seconds. The light intensity generated by either of the two lamps was measured using a Macam Quantum Photometer (model number: Q101-1) for a distance of 13.5cm. It was found that the intensity of the 500 Watt lamp is 1,090 μMoles $m^{-2}s^{-1}$ whilst that of the 1,000 Watt lamp is 9,007 μmoles $m^{-2}s^{-1}$, approximately eight times as much. Thus, as their name implies, photosensitized reactions are primarily dependent on the intensity of the light source used.

Although the effect is accompanied by an increase in protein cross-linking, which in this case is an unwanted side reaction, as will be seen in later experiments, conditions were optimized so that the latter reaction was decreased to an acceptable level. In photosensitized radioactive labelling, the main

Table 3-(i): Dependence of Radioactive Incorporation on Light Source Used.

LIGHT SOURCE* (Watts)	INCORPORATION (dpm/pmole fibrinogen)
500	52.0
1000	696.6

The above results represent the mean of five experiments.
In all cases the standard error was $\leq \pm 9\%$

* Irradiation time = 60 seconds

objective is that "P-[^{3}H]trp" should be the predominant end-product where P = protein and [^{3}H]trp = tritiated tryptophan.

This can be done, for example, by selecting a shorter irradiation time which in turn also decreases the possibility of any adverse effects occuring, such as denaturation of the protein by heat. The temperature of the reaction mixture was measured after 30 seconds and 60 seconds exposure by the 1000 Watt lamp. The highest temperatures recorded after the latter irradiation times were 19°C and 32°C respectively, where the initial temperature prior to illumination was 10°C in both cases. As will be seen in this chapter, a fixed irradiation time no longer than 30 seconds was selected for all future experiments.

Another option is to minimize the concentration of protein used, bearing in mind that it is necessary to have sufficient protein present for: (a) subsequent analysis on SDS-polyacrylamide, or Laemmli, gels after labelling, and (b) digestion of the labelled fibrinogen. Consequently, unless specified, the concentration of protein used in each photolabelling experiment was 1.32nM (0.3mg/ml).

Initial attempts to cross-link fibrinogen in the Tris/$CaCl_2$ buffer (pH = 7.5), using a 500 Watt lamp also proved to be futile, even though experimental conditions such as the following (overleaf) were varied:

(a) time of irradiation (from 25-90 seconds),

(b) protein concentration (from 2-6 nM),

(c) fibrinogen derived from various purifications,

(d) distance of light source from sample,

(e) fluorescein prepared from different batches.

The only suggestion that cross-linking had occured resulted when 20µl of the free radical catalyst accelerator, TEMED, were added to the sample immediately prior to irradiation. This generated some fibrinogen dimer and a very small amount of the trimer species, as observed on 3% SDS-polyacrylamide gels. On examination of the reduced protein on Laemmli gels (3:7½%), some high molecular weight cross-linked bands were observed, situated above the Aα band. However, when a Philips Watastar 1000 lamp was used, the evidence that cross-linking had occurred became very clear as will be seen in all future experiments. The final concentration of protein used in each photosensitized cross-linking experiment was 2.94nM (0.67mg/ml).

Removal of Fluorescein and Excess [^{3}H]tryptophan Following the Photolabelling Reaction.

Cross-linked samples could be run directly on gels without prior removal of the heterocyclic dye, fluorescein (molecular weight, 332). Due to its comparatively low molecular weight, the latter is removed from the protein sample simply by the electrophoretic technique itself. However a different situation presented itself in photolabelling reactions since it was found

that excess, unbound, radioactive [^{3}H]tryptophan could not be removed simply by gel electrophoresis. Two possibilities for the removal of excess label after the reaction was carried out were investigated. These were:

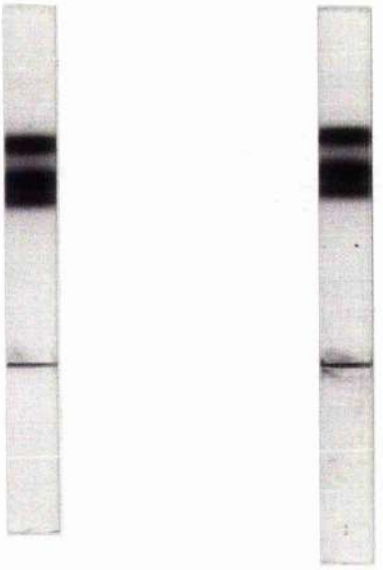
(i) dialysis, or,

(ii) gel filtration using a Sephadex G-25 column.

As mentioned previously, it is more advantageous to use a low protein concentration in photolabelling experiments so that minimal cross-linking occurs. However, in the initial pilot experiments a final concentration of 0.42mg/ml (1.85nM) fibrinogen was labelled per irradiation experiment.

In gel filtration, the labelled protein was recovered in the various fractions eluting off the Sephadex G-25 column. Following the simultaneous application of five photolabelled fibrinogen samples onto the column, a maximum yield of 0.13mg/ml was recorded in the peak protein fraction. Such recovery concentration meant that the fraction had to be freeze-dried in order to run gels. In view of the lability of the Aα-chains and low protein recovery following freeze-drying, a method where this step is not necessary was preferable.

With dialysis, a concentration of approximately 0.3mg/ml protein per sample dialysed was recovered. Thus no freeze-drying was necessary and gels could be run directly after dialysis. Figure 3-6 shows two gels (5% acrylamide) of reduced pre- and



Pre-dialysis *Post-dialysis*

Figure 3-6: SDS-polyacrylamide gels (5% acrylamide) of reduced fibrinogen before and after dialysis.

post-dialysis fibrinogen which had been dialysed for 24 hours at 4°C. As can be seen from these gels, the integrity of all bands has been preserved.

A study to investigate the efficacy of dialysis in the removal of unbound [^{3}H]tryptophan was also carried out. The radioactive photosensitized labelling procedure was carried out as described in the previous chapter, on a sample containing no protein (irradiation time = 60 seconds). The radioactive mixture was then dialysed for 24 hours at 4°C. Samples were removed prior to dialysis and also 3 hours, 17 hours and 24 hours after dialysis had been initiated. The radioactivity contained in a 1:100 dilution of each sample was then measured (dpm) and the results can be seen in Table 3-(ii), under the heading "BLANK".

As can be seen from this Table, dialysis is an efficient way of removing unbound radioactivity; even though this method does not remove 100% of the initial radioactivity, it is 99.7% effective. The residual radioactivity, although minimal compared to pre-dialysis levels, may be due to adherence of the [^{3}H]tryptophan to the dialysis sac.

The experiment was then repeated under the same conditions however this time the sample contained fibrinogen (concentration = 0.31mg/ml). After 24 hours, it was found that a higher level of residual radioactivity resulted, compared to the blank experiment, indicating that [^{3}H]tryptophan must have bound to

Table 3-(ii): The Removal of Excess [^{3}H]tryptophan By Dialysis.

	BLANK	+ PROTEIN
Sample	Radioactivity (dpm)	Radioactivity (dpm)
predialysis	115,630	114,027
+ 3 hours	1,885	-
+ 17 hours	593	-
+ 24 hours	450	2,844

The above results represent the mean of three experiments.
In all cases the standard error was $\leq \pm 3.5\%$

the protein. It should be noted, however, that since not all of the unbound radioactivity is removed by dialysis, calculations regarding the actual amount of radioactive label binding to the protein must be based on data derived from the solubilization of radioactive gels and not simply from post-dialysis readings of protein solutions.

Irradiation Time

Initial radioactive photosensitized labelling experiments on fibrinogen were carried out for irradiation times of 60 seconds and 30 seconds according to the method described in the previous chapter, in the Tris/$CaCl_2$ buffer, (pH = 7.5), at a final protein concentration of 1.85nM.

Fibrinogen samples from each of the irradiation times were then run on 3% SDS-polyacrylamide gels; examples of densitometer scans can be seen in Figures 3-7 (a) and (b). As can be seen from the latter, the proportion of fibrinogen dimer and trimer is greater at 60 seconds than at 30 seconds. A small proportion of tetramer is also present at 60 seconds (this can also be seen in Table 3(iii)). The incorporation (dpm per pmole fibrinogen) was measured by solubilization of the radioactive gels run and it was found that the incorporation at 30 seconds was approximately half that resulting from a 60 second irradiation time. The total amount of polymer at 30 seconds (15.8%) is also approximately half that obtained at 60 seconds (31.2%); however

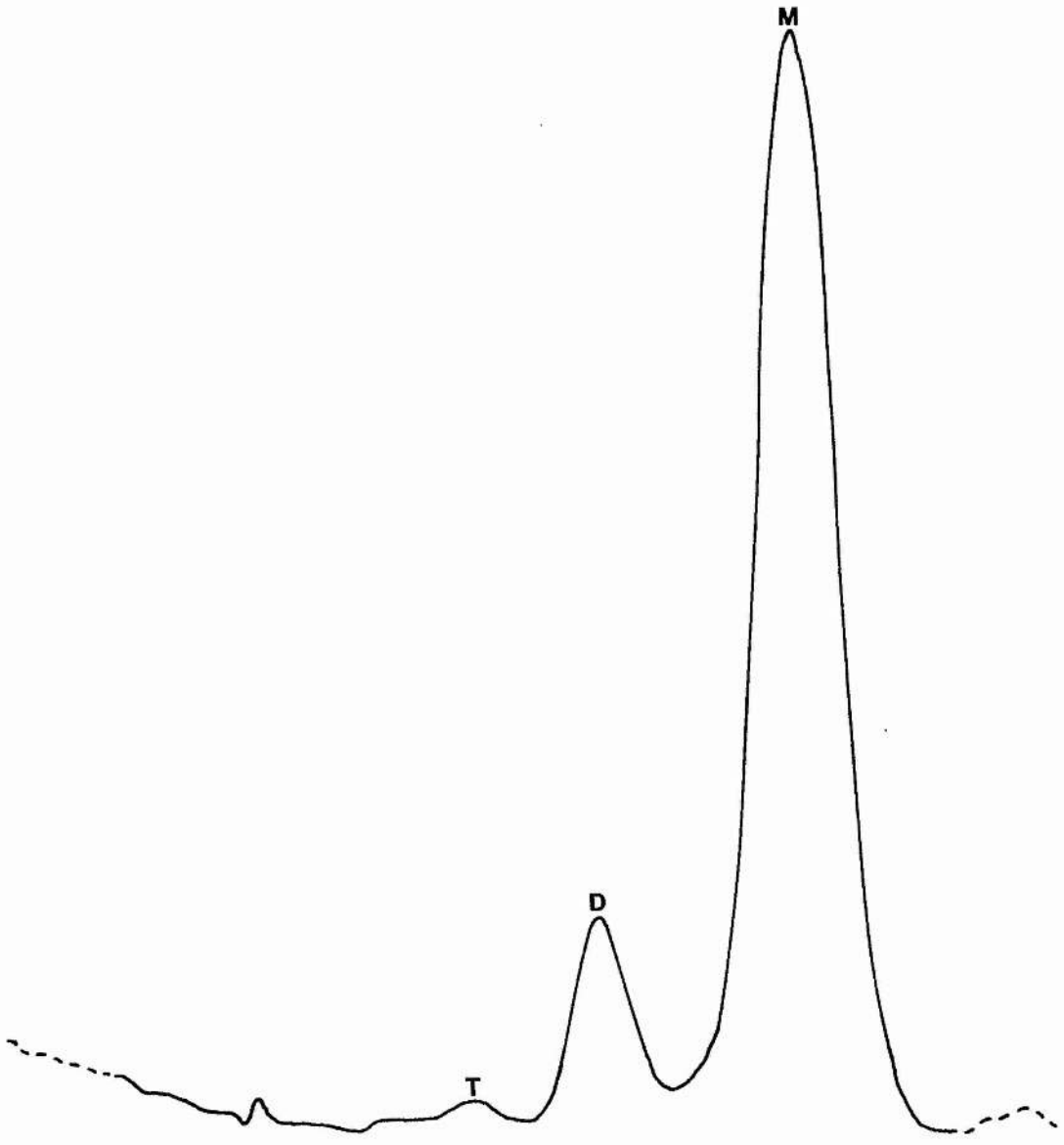


Figure 3-7(a): Densitometer scan of an SDS-polyacrylamide gel (3% acrylamide) for a fibrinogen sample irradiated for 30 seconds.

M = monomer

D = dimer

T = trimer

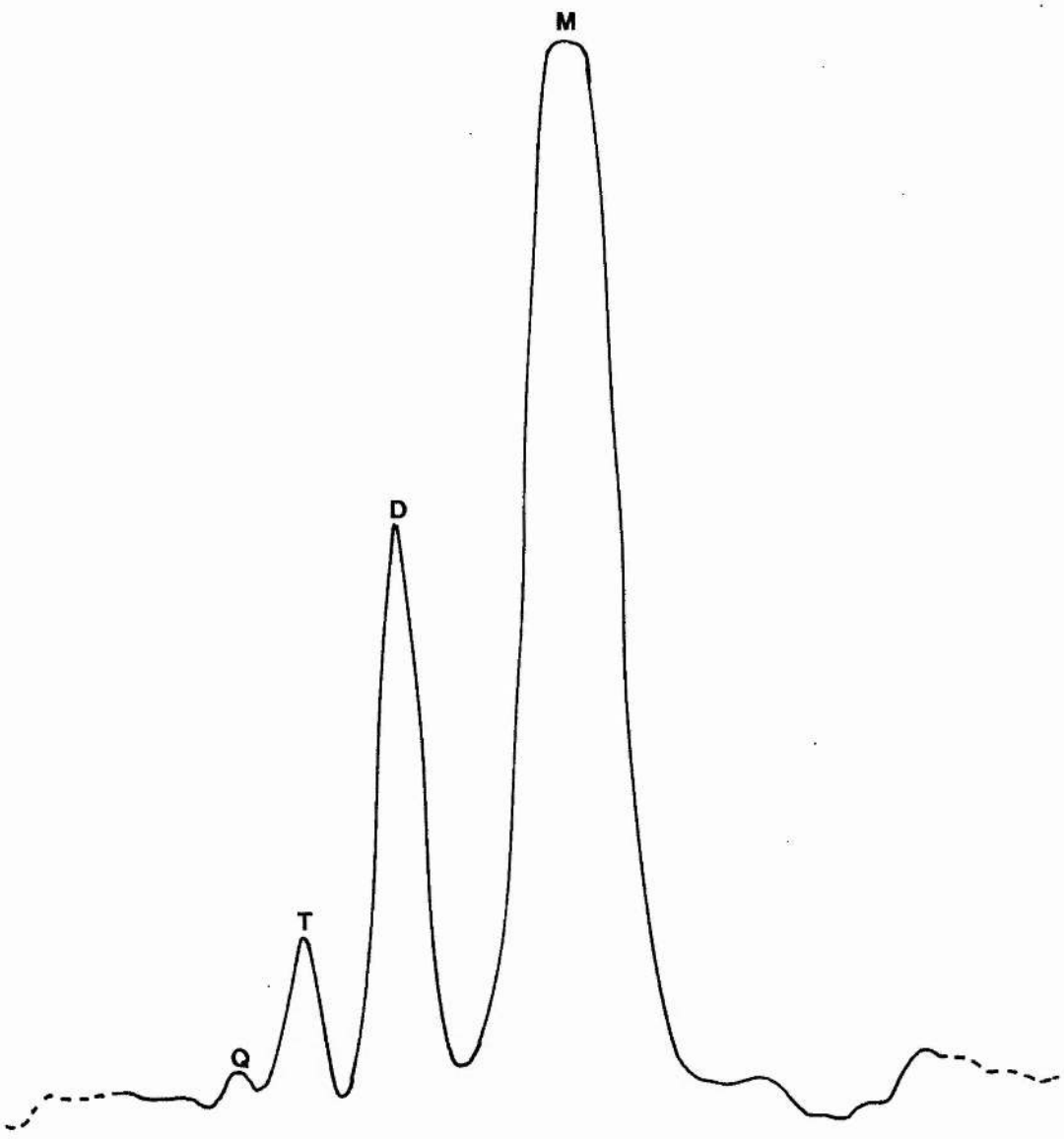


Figure 3-7(b): Densitometer scan of an SDS-polyacrylamide gel (3% acrylamide) for a fibrinogen sample irradiated for 60 seconds.

M = monomer

D = dimer

T = trimer

Q = tetramer

Table 3-(iii): Photosensitized Radioactive Labelling at 30 seconds and 60 seconds.
(Buffer used: 0.05M Tris-HCl, 2mM $CaCl_2$, pH = 7.5)

IRRADIATION TIME (seconds)	%MONOMER	%DIMER	%TRIMER	%TETRAMER	%INCORPORATION (dpm/pmole fibrinogen)
30	84.2	12.9	2.9	-	297.0
60	68.8	22.2	7.2	1.8	696.6

The above results represent the mean of five experiments.
In all cases the standard error was $\leq \pm 7\%$.

the possibility of decreasing the irradiation time further so as to minimize cross-linking was investigated.

Consequently the aim of the next experiment was to investigate incorporation and cross-linking patterns of fibrinogen at various times of irradiation namely 10, 20, 30, 45, and 60 seconds. From the latter, a suitable irradiation time for future experiments was selected.

A graph of residual radioactivity in the dialysed samples (following irradiation) versus irradiation time can be seen in Figure 3-8. This demonstrates that the longer the irradiation time, the greater the amount of radioactivity present in the dialysate and thus bound to the protein.

3% SDS-polyacrylamide gels were then run for the non-reduced fibrinogen samples (see Figure 3-9). As time of irradiation is increased, the appearance of dimers, trimers and even tetramers (at 60 seconds) of fibrinogen result due to intermolecular cross-linking. Figure 3-10 shows two graphs of % fibrinogen monomer/polymer versus irradiation time (seconds) which are the result of scanning the 3% gels using a densitometer. The graphs show that a decrease in the amount of fibrinogen monomer with time is coupled by a proportional increase in the amount of polymer present. From such graphs it was concluded that irradiation times above 30 seconds can be discarded since the amount of cross-linking which results is too

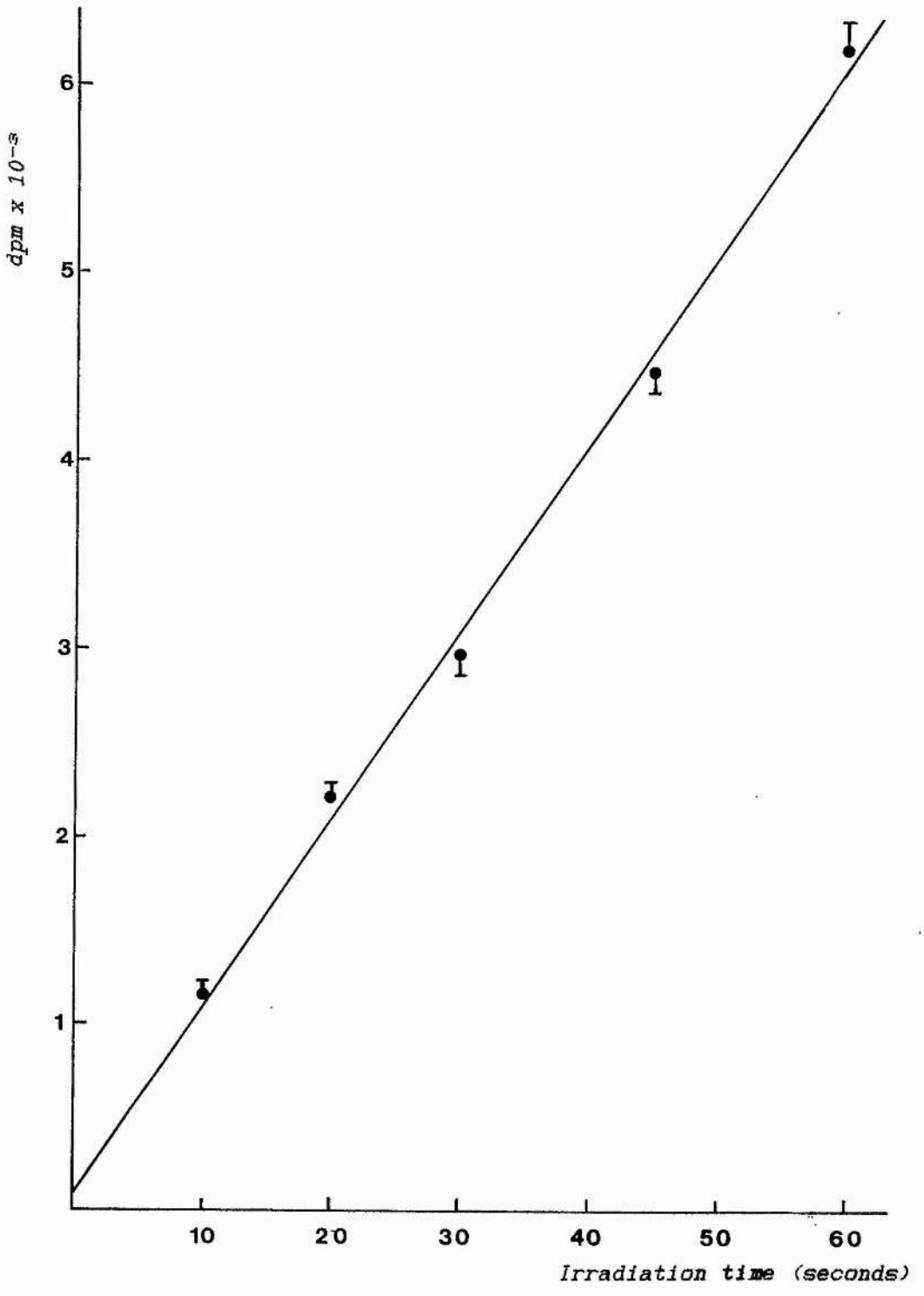


Figure 3-8: Graph of residual radioactivity in dialysed samples (dpm x 10^{-3}) versus irradiation time (seconds).

The points represent the mean of two experiments.

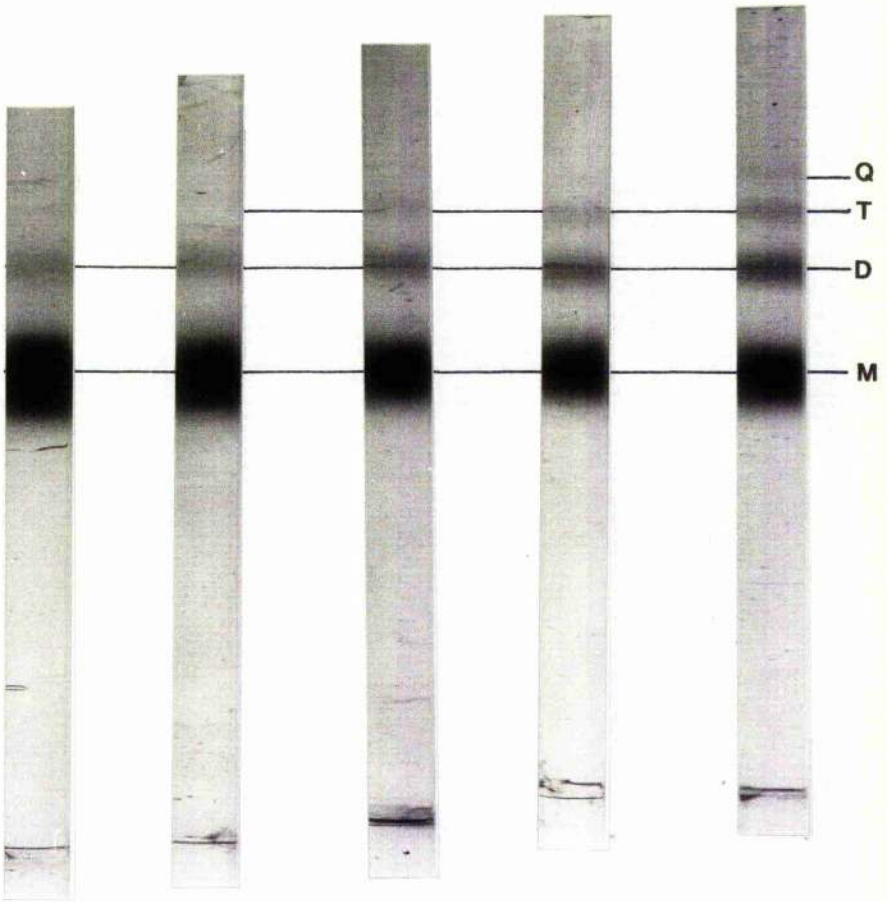


Figure 3-9: SDS-polyacrylamide gels (3% acrylamide) of fibrinogen irradiated for 10 - 60 seconds in 0.05M Tris-HCl, pH 7.5, containing 2mM $CaCl_2$.

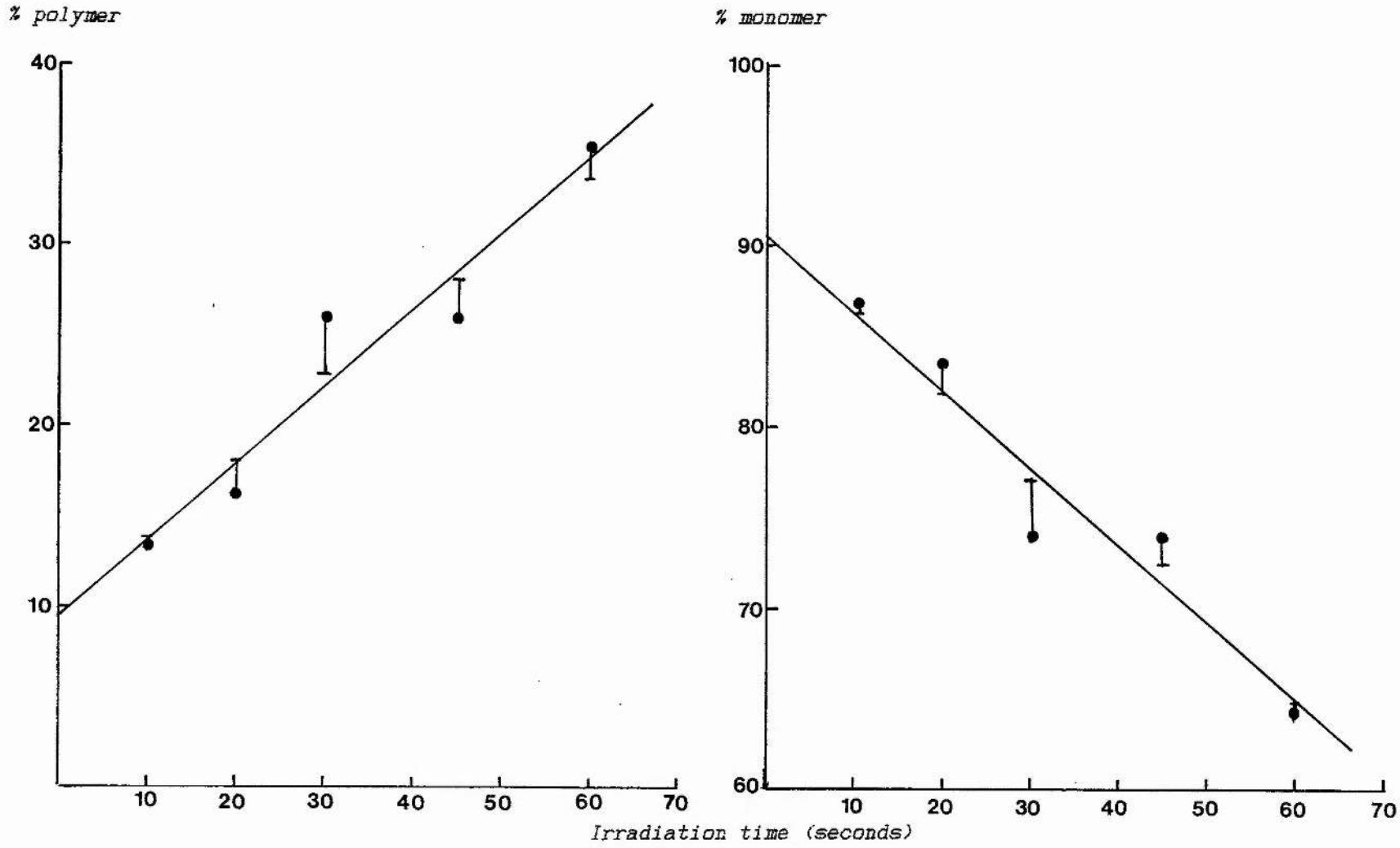


Figure 3-10: Graphs of (a) % polymer and (b) % monomer versus irradiation time (seconds).

The points represent the mean of two experiments.

high. In fact, an irradiation time of 20 seconds, to be used in all future photosensitized labelling experiments was selected at this stage. This irradiation time represents the best compromise, where the degree of cross-linking is comparatively small and the level of incorporation is sufficiently high for analysis. Also on the basis of these results, an irradiation time of 30 seconds was selected to be used in all photosensitized cross-linking experiments.

It is also interesting to note that if the graphs are extrapolated back to time t = 0, the initial amount of monomer present (under these experimental conditions) is approximately 91.5%, whilst the amount of polymer present is 8.5%. This suggests that some of the fibrinogen is already present as polymer in its native state. The latter complexes would not be detected on SDS-polyacrylamide gels due to the SDS/urea in the gel system which would break down any such intermolecular bonds present.

The radioactivity of the gel slices was then measured after solubilization and the incorporation patterns were noted and compared for various irradiation times. From Figure 3-11 it can be seen that:

$$\text{Incorporation} \propto \text{Irradiation time.}$$

It should also be noted (from this graph) that the degree of radioactive incorporation for the selected irradiation time of 20 seconds is sufficient for analysis.

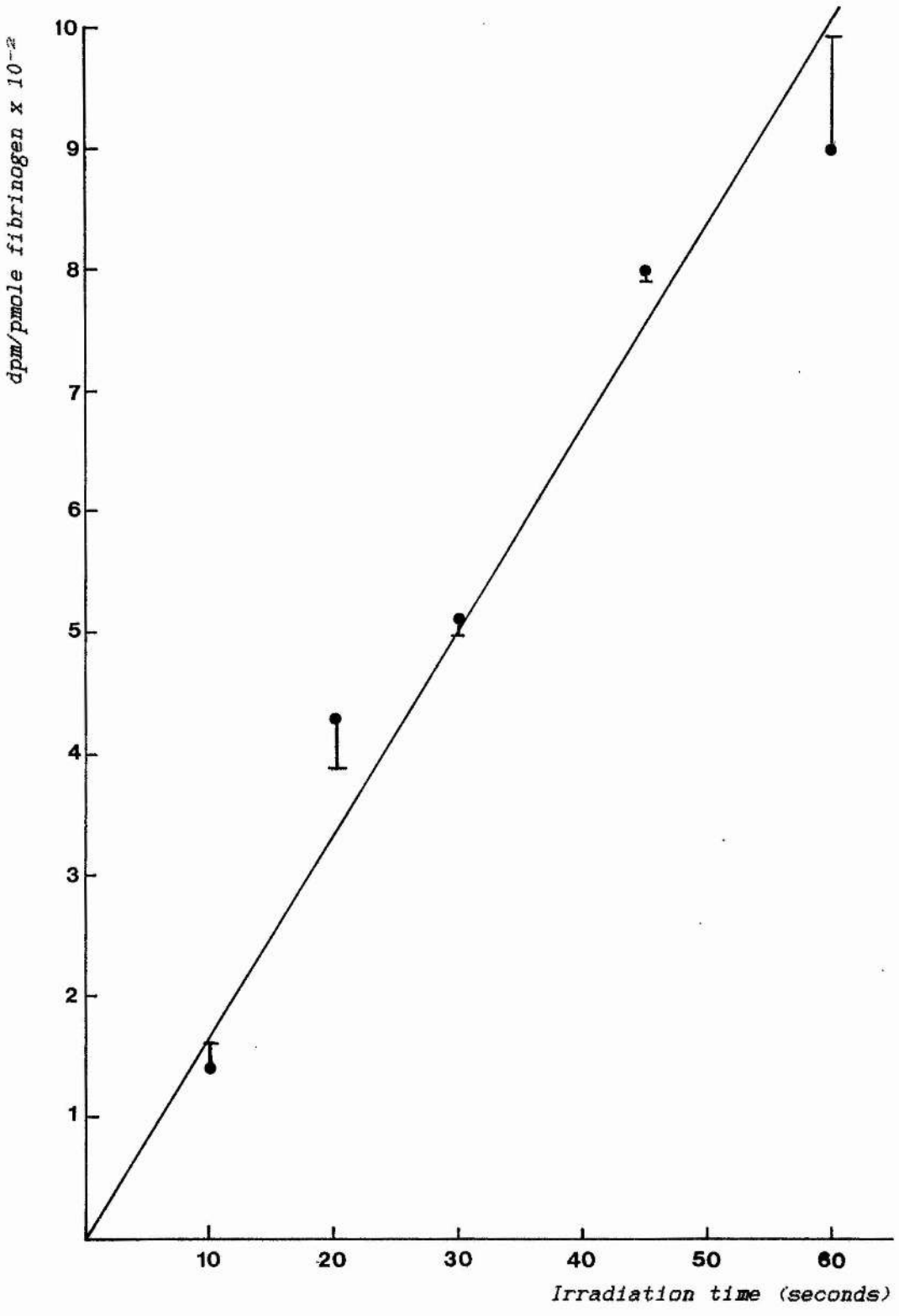


Figure 3-11: Graph of radioactive incorporation (dpm/pmole fibrinogen x 10^{-2}) versus irradiation time (seconds).

(Buffer: 0.05M Tris-HCl, pH 7.5, containing 2mM $CaCl_2$).

The points represent the mean of two experiments.

Section (3): Buffer Effects

There has been much controversy concerning the conformation of fibrinogen with several models being put forward ranging from a trinodular structure (Hall and Slayter, 1959) to a more globular model (Koppel, 1966).

Although there are a number of possible reasons for the plethora of models, such as different methods of fibrinogen preparation or artefacts of the techniques used to examine conformation, an attractive proposition is that of Mueller and Burchard (1978). These authors suggest that fibrinogen is a flexible molecule dependent on the particular enviroment.

Thus, once the main problems in the experimental methodology had been overcome, the next step was to test the above premise by investigating the photosensitized labelling and cross-linking of both fibrinogen and its plasmin derivatives, fragments D and E, under different conditions, with particular reference to the influence of calcium.

(i) - The aim of the first experiment was to investigate the effect a buffer of different composition and pH to the one used previously (i.e. 0.05M Tris-HCl, pH 7.5, containing 2mM $CaCl_2$) would have on the incorporation and cross-linking patterns of fibrinogen at various irradiation times. The last experiment in Section (2) was thus repeated, however in this case the selected

buffer was 0.13M NaCl, 6.7mM sodium phosphate, pH 7.0 (from here on referred to as the NaCl/phosphate buffer). This particular buffer was chosen since, apart from being completely different in composition and pH to the initial buffer used, it contains no added calcium and is also the buffer used by Hemmendorff et al (1981), on whose photosensitized labelling technique this method is based.

It should be noted that although the NaCl/phosphate buffer contains no added calcium, no rigorous attempts were made to remove calcium from the fibrinogen. In fact, atomic absorption measurements for the calcium content of such 'calcium-free' buffers showed that these do indeed contain traces of calcium ions (Ross, 1982), example:

(i) 0.05M Tris, pH 7.5 : contains 1.90μM Ca^{++}

(ii) 0.15M NaCl : contains 3.75μM Ca^{++}

(iii) 0.05M Tris, 0.15M NaCl, pH 7.5 : contains 5.00μM Ca^{++}

Thus the calcium high affinity sites can assumed to be occupied at "zero" calcium concentrations used in these and following experiments.

After irradiation and dialysis, 3% and 5% SDS-polyacrylamide gels were run for non-reduced and reduced fibrinogen samples respectively. (See Figures 3-12 (a) and (b)). Although, as can be seen from the 3% gels, there is an increase in the amount of polymer formed with irradiation time, this increase is not as marked as it was in the Tris/$CaCl_2$ buffer. The appearance of

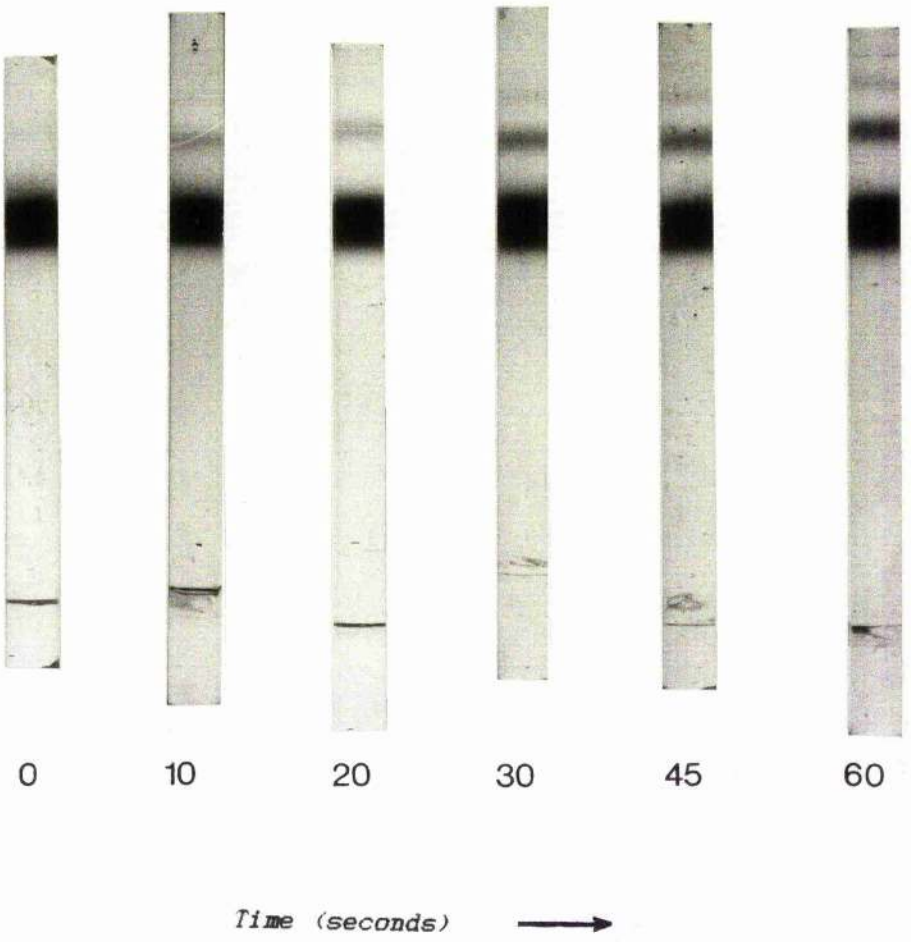


Figure 3-12(a): SDS-polyacrylamide gels (3% acrylamide) of fibrinogen irradiated for 0 - 60 seconds in 0.13M NaCl, 6.7mM sodium phosphate, pH 7.0.

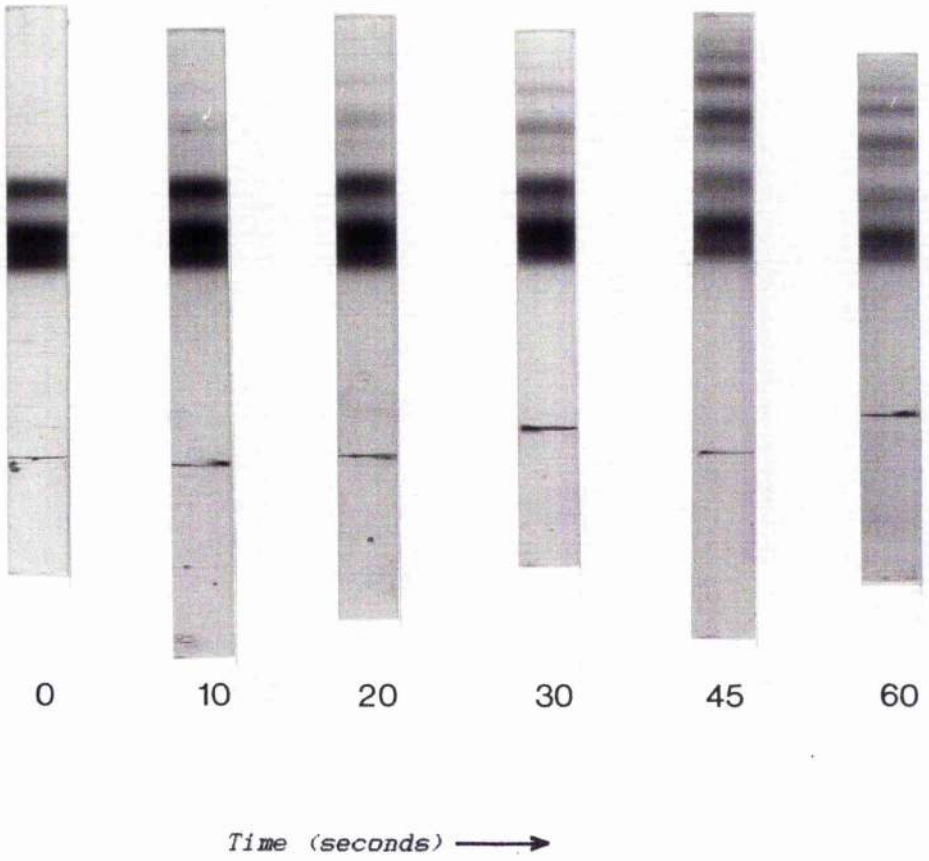


Figure 3-12(b): SDS-polyacrylamide gels (5% acrylamide) of fibrinogen irradiated for 0 - 60 seconds in 0.13M NaCl, 6.7 mM sodium phosphate, pH 7.0, then reduced.

trimer is only just apparent at 30 seconds and no tetramer is present at 60 seconds. This contrasts with the results in the previous section where appearance of trimer, albeit minimal, occurs at 20 seconds and where tetramer is clearly seen on the 60 second gel. These results suggest that fibrinogen is a molecule whose conformation is influenced by its enviroment. It is also possible that the increased amount of intermolecular cross-linking seen in the Tris/$CaCl_2$ buffer could be due to the molecule adopting a more open conformation under these conditions.

The radioactivity of the gel slices was then measured after solubilization and the incorporation plotted for the various irradiation times (see Figure 3-13). The relationship between these two variables is still linear, however it should be noted that the overall level of incorporation into fibrinogen, as measured in the two buffers, is different; this is significantly higher in the NaCl/phosphate buffer.

(ii) - The next step was to investigate the photosensitized labelling of a derivative molecule of fibrinogen, namely the plasmin-derived core fragment E, in the two different buffers (irradiation time = 20 seconds, concentration = 1.85nM).

The non-reduced, labelled, fragment E was run on 5% SDS-polyacrylamide gels and the protein bands solubilized. The incorporation resulting in the two buffers can be seen in

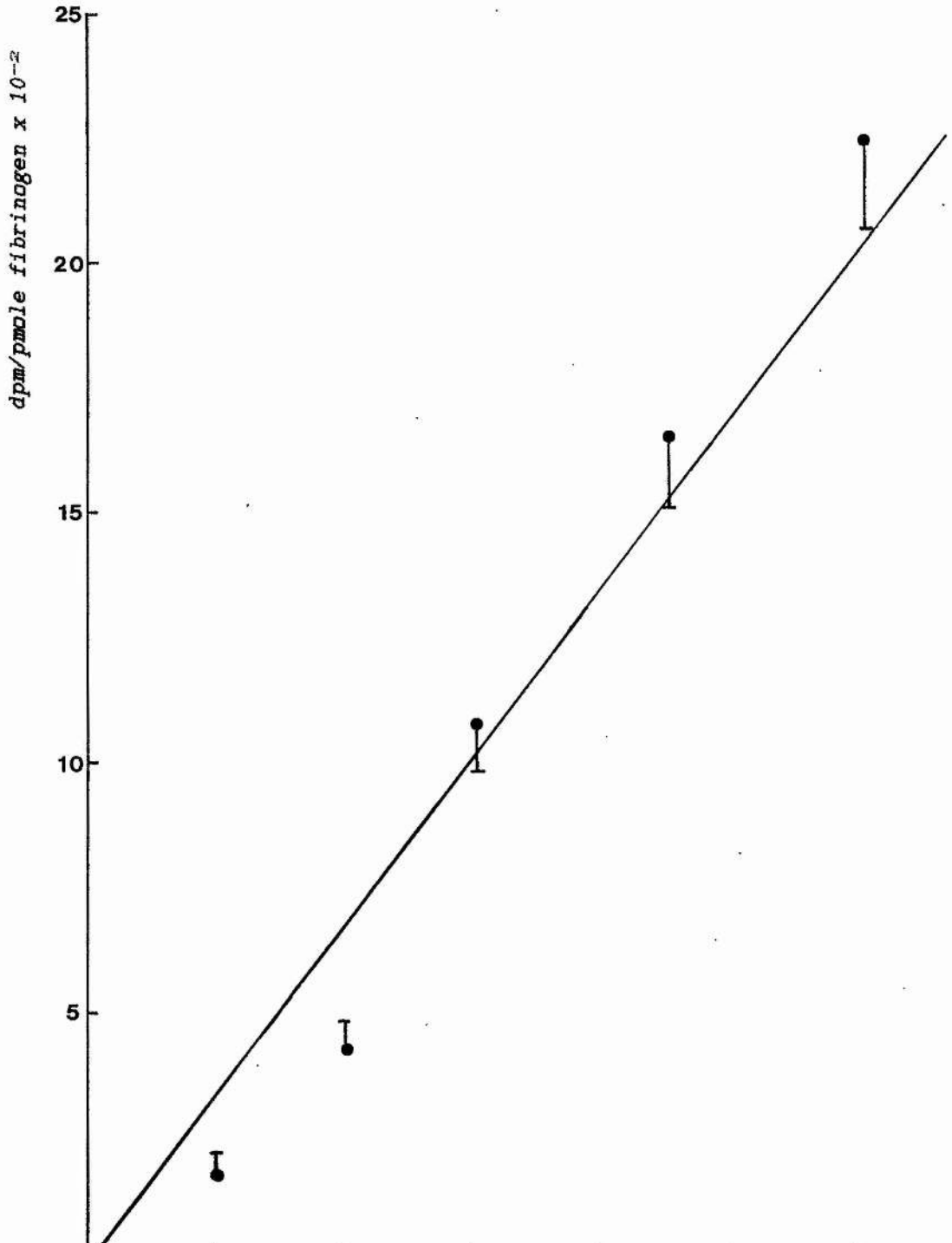


Figure 3-13: Graph of radioactive incorporation (dpm/pmole fibrinogen x 10^{-2}) versus irradiation time (seconds).

(Buffer: 0.13M NaCl, 6.7mM sodium phosphate, pH 7.0).

Table 3-(iv). Incorporation levels in the NaCl/phosphate buffer are approximately four times those found in the Tris/$CaCl_2$ buffer.

Four possibilities as to why this should happen were investigated:-

(a) - the photolabelling reaction proceeds via free radical intermediates; the depressed incorporation in the Tris/$CaCl_2$ buffer could be attributed to a possible free radical scavenger effect of Tris,

(b) - the pH of the buffer affects the protein's conformation and thus incorporation levels,

(c) - the pH of the buffer affects the rate of the reaction, or

(d) - the conformation of the protein is influenced by calcium which is only present in one of the buffers.

(iii) - In order to investigate whether Tris has an inhibitory effect on the labelling reaction and consequent radioactive incorporation, fragment E was labelled in the two different buffers used previously, but this time made up to the same pH, i.e. pH 7.5 (This was done to eliminate any pH effects). The resulting incorporation into fragment E can be seen in Table 3-(v).

Even though the pH of both buffers is 7.5, incorporation in the Tris/$CaCl_2$ buffer is still much lower. These results suggest that the presence of Tris is a factor contributing towards this

Table 3-(iv): Incorporation of Radioactive Label Into Fragment E.

BUFFER	INCORPORATION (dpm/pmole Fragment E)
0.05M Tris-HCl, 2mM $CaCl_2$, pH = 7.5	65.0
0.13M NaCl, 6.7mM sodium phosphate, pH = 7.0	281.7

The above results represent the mean of six experiments.
In all cases the standard error was $\leq \pm 6\%$, and the significant difference, p, was ≤ 0.001.

Table 3-(v): Incorporation of Radioactive Label Into Fragment E at pH 7.5.

BUFFER	INCORPORATION (dpm/pmole Fragment E)
0.05M Tris-HCl, 2mM $CaCl_2$, pH = 7.5	65.0
0.13M NaCl, 6.7mM sodium phosphate, pH = 7.5	187.9

The above results represent the mean of six experiments.
In all cases the standard error was $\leq \pm 6.5\%$, and the significant difference, p, was ≤ 0.001.

decreased level of incorporation by its action as a free radical scavenger. In fact this was also seen later, from results of cross-linking experiments done in the two buffers. However, it should be noted that Tris may simply be one of several factors contributing to this difference since the buffers under comparison are different in several other respects, as well as the Tris content.

It was also noted that the incorporation into fragment E was different for the two identical NaCl/phosphate buffers made up at different pH's (see Tables 3-(iv) and 3-(v)). Thus, the next experiment was designed to answer the following question:- Does the pH of the buffer affect the incorporation of radioactivity into fragment E by altering the rate of the reaction, or the conformation of the molecule?

(iv) - 8M urea was used to eliminate differences in protein structure at the different pH's due to its denaturing effect on the molecule. Thus, radioactive incorporation should be the same for each pH unless the reaction is pH-sensitive in which case incorporation at the various pH's will differ.

The buffer used was 0.13M NaCl, 6.7mM sodium phosphate, 8M urea, made up at pH = 7.0, 7.5 and 8.0 respectively. The results can be seen in Table 3-(vi) and Figure 3-14, where the latter represents the radioactivity profile along each of the three gels representing the different pH's.

Table 3-(vi): Radioactive Incorporation into Fragment E at pH 7.0, 7.5, and 8.0, in 8M Urea.

Buffer used : 0.13 M NaCl,
6.7mM sodium phosphate,
8M Urea.

pH	INCORPORATION (dpm/pmole fragment E)
7.0	22.6
7.5	63.4
8.0	131.5

The above results represent the mean of two experiments.
In all cases the standard error was $\leq \pm 12\%$, and the significant difference, p, was ≤ 0.05.

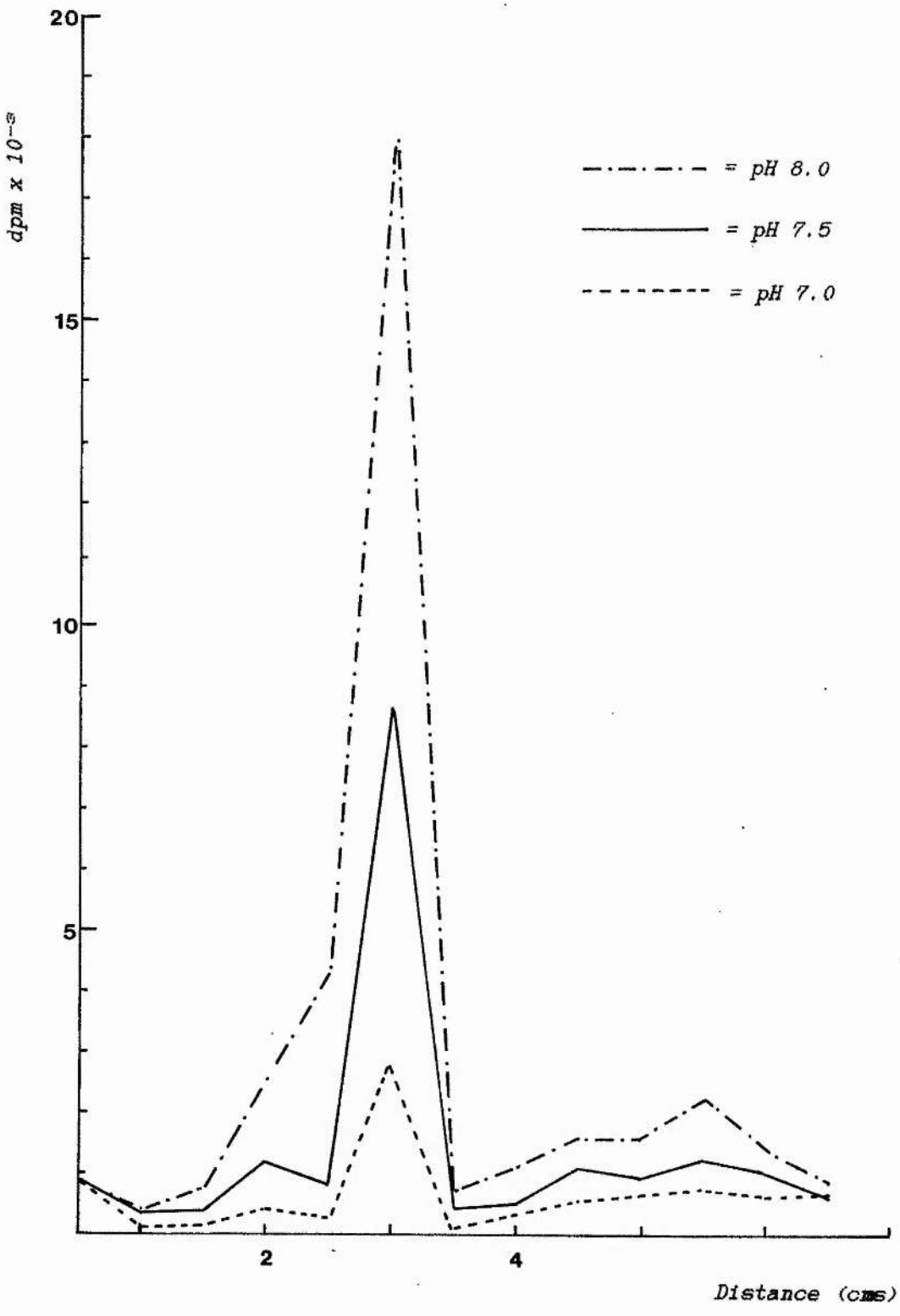


Figure 3-14: Graph of radioactive incorporation (dpm x 10^{-3}) versus distance from top of gel (cms).

(Gel acrylamide concentration = 5%).

The results clearly indicate that the rate of the reaction is dependent on pH. It is therefore important to select and work at one specific pH so as to eliminate such effects. The pH chosen for the rest of the experiments was pH 7.5.

The results also show and confirm that the photosensitized labelling technique is indeed conformation-dependent. This is so since the level of incorporation into fragment E, at pH 7.0 and pH 7.5 respectively, is different to that which resulted when the same experiment was repeated in the presence of 8M urea (compare results in Tables 3-(iv) and 3(v) with those in Table 3(vi)).

(v) - The final photosensitized labelling experiment in this section was carried out on fibrinogen in three buffer systems, namely :-

(A) 0.13 M NaCl, 6.7mM sodium phosphate, pH 7.5,

(B) 0.05M Tris-HCl, pH 7.5, and

(C) 0.05M Tris-HCl, 2mM $CaCl_2$, pH 7.5.

The aim of this experiment was to investigate whether the protein's conformation is directly influenced by the presence of calcium. Thus buffer (B), (from here on referred to as the Tris buffer), was used for direct comparison with the calcium-containing buffer (C).

Intact fibrinogen was labelled for 20 seconds and the incorporation of surface label onto the protein and its constituent chains was measured. The results for non-reduced fibrinogen are shown in Table 3-(vii) whereas those for the reduced chains can be seen in Table 3-(viii).

Table 3-(vii) illustrates once again that less overall incorporation occurs in both Tris-containing buffers compared to the NaCl/phosphate buffer. However, there is a difference in incorporation between the two Tris-containing buffers, where a higher level of incorporation occurs in the Tris/$CaCl_2$ buffer.

The results presented in Table 3-(viii) show that the distribution of label incorporated into the three constituent chains of fibrinogen is similar in ratio for buffers (A) and (B), but not for buffer (C). This suggests that conformational change is a factor contributing towards the difference in incorporation. In the Tris/$CaCl_2$ buffer the Aα-chain takes up almost as much label as the other two chains combined, whilst in both the Tris and NaCl/phosphate buffers the Aα and γ-chains are equally exposed. The Bβ-chain is the least exposed in all media.

All this suggests that another component in the buffer affects incorporation into fibrinogen and thus the molecule's conformation. Both the NaCl/phosphate and Tris buffers contain no added calcium. Consequently the effect of calcium on the molecule was further investigated. (See Results, Section (4)).

Table 3-(vii): The Photosensitized Radioactive Labelling of Intact Fibrinogen.

BUFFER	INCORPORATION (dpm/pmole fibrinogen)
(A) 0.13M NaCl, 6.7mM sodium phosphate, pH = 7.5	1041.8
(B) 0.05M Tris-HCl, pH = 7.5	97.1
(C) 0.05M Tris-HCl, 2mM $CaCl_2$, pH = 7.5	225.5

The above results represent the mean of six experiments.
In all cases the standard error was $\leq \pm 5\%$, and the significant difference, p, was ≤ 0.001.

Table 3-(viii): Radioactive Labelling of The Aα, Bβ, and γ-Chains of Fibrinogen.

BUFFER	Aα	Bβ	γ
(A) 0.13M NaCl, 6.7 mM sodium phosphate, pH = 7.5	1.7	1	1.6
(B) 0.05 M Tris-HCl, pH = 7.5	1.6	1	1.4
(C) 0.05M Tris-HCl, 2mM $CaCl_2$, pH = 7.5	2	1	1.1

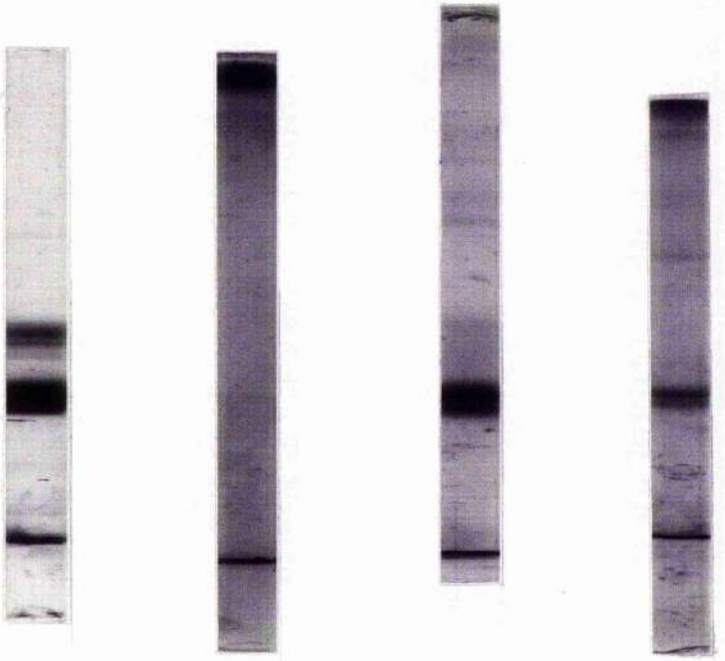
The above results are expressed as the ratio of label incorporated by each chain to that incorporated by the Bβ-chain.
The figures represent the mean of six experiments. In all cases the standard error was $\leq \pm 8\%$, and the significant difference, p, was ≤ 0.001.

(vi) - The next step, however, was to investigate the photosensitized cross-linking patterns of fibrinogen in the three different buffers (ie. A, B, and C) shown in Tables 3-(vii) and 3-(viii). The method used is the one decribed in the previous chapter.

Both Laemmli (3:7½%) and SDS-polyacrylamide gels (5% and 3% acrylamide respectively) of the reduced and non-reduced cross-linked fibrinogen samples were run (see Figures 3-15 (a), (b), and (c)). Control gels of uncross-linked fibrinogen were also run in order to identify which bands are taken up by cross-linking.

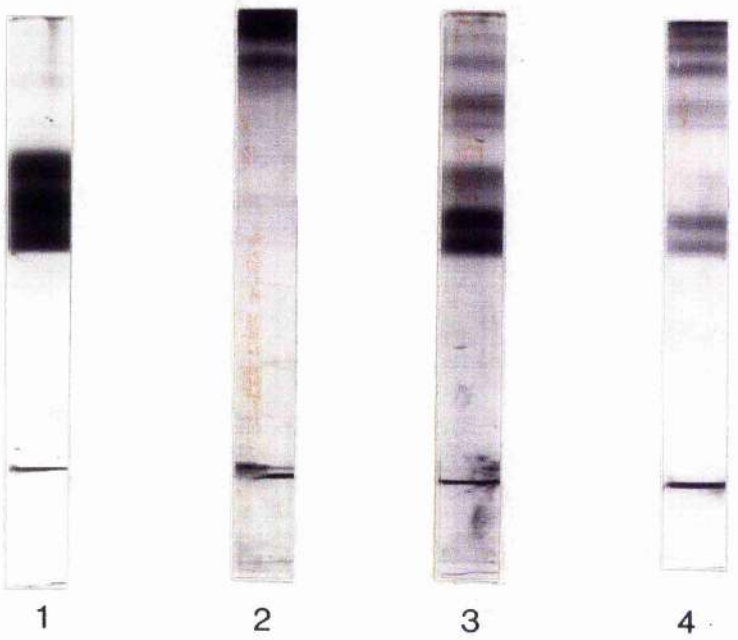
Figures 3-15 (a) and (b) show that extensive cross-linking occured in the NaCl/phosphate buffer, where all three chains have been taken up in cross-links. However, in the Tris/$CaCl_2$ buffer, cross-linking was less extensive. This difference was initially thought to be entirely due to the free radical scavenger effect of Tris. However, on examination of the gels for fibrinogen cross-linked in the Tris buffer alone, it was found that cross-linking was less extensive in this gel compared to the Tris/$CaCl_2$ gel.

On examination of the 3% gels (see Figure 3-15 (c)) it was seen that more intermolecular cross-linking occurs in the Tris/$CaCl_2$ buffer compared to that which occurs in both the NaCl/phosphate and Tris buffers respectively. All of this, plus



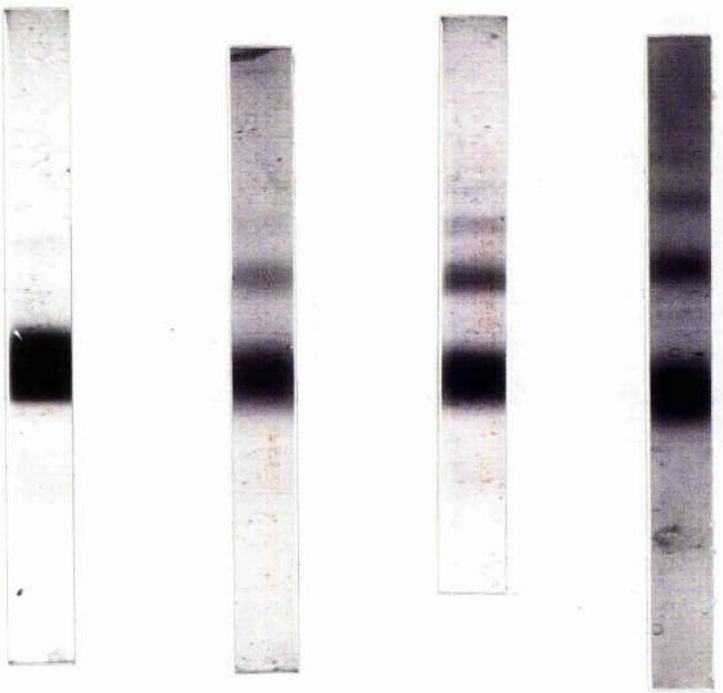
1 = Control (ie. no cross-linking)
2 = Buffer (A): 0.13M NaCl, 6.7mM sodium phosphate, pH 7.5
3 = Buffer (B): 0.05M Tris-HCl, pH 7.5
4 = Buffer (C): 0.05M Tris-HCl, 2mM $CaCl_2$, pH 7.5

***Figure 3-15(a)**: Laemmli gels (3:7½ %) of fibrinogen samples cross-linked under different solvent conditions, then reduced.*



1 = Control (ie. no cross-linking)
2 = Buffer (A): 0.13M NaCl, 6.7mM sodium phosphate, pH 7.5
3 = Buffer (B): 0.05M Tris-HCl, pH 7.5
4 = Buffer (C): 0.05M Tris-HCl, 2mM $CaCl_2$, pH 7.5

Figure 3-15(b): *SDS-polyacrylamide gels (5% acrylamide) of fibrinogen samples cross-linked under different solvent conditions, then reduced.*



1 = Control (ie. no cross-linking)
2 = Buffer (A): 0.13M NaCl, 6.7mM sodium phosphate, pH 7.5
3 = Buffer (B): 0.05M Tris-HCl, pH 7.5
4 = Buffer (C): 0.05M Tris-HCl, 2mM $CaCl_2$, pH 7.5

Figure 3-15(c): ***SDS-polyacrylamide gels (3% acrylamide) of fibrinogen samples cross-linked under different solvent conditions.***

earlier incorporation results from the photosensitized labelling experiments, suggests that the molecule adopts a more open conformation in the presence of calcium. This phenomenom was further investigated (Results, Section (4)).

Full plasmin digestion of the cross-linked samples, plus that of a control, was then carried out according to the method outlined in the previous chapter. The aim of this experiment was to investigate whether or not the D and E domains are involved in the conformational changes of fibrinogen which give rise to the different cross-linking patterns in the three buffers.

5% SDS-polyacrylamide gels of the non-reduced samples were run after a digestion time of six hours at 37°C. The control gel pattern (resulting from the digestion of uncross-linked fibrinogen) showed two protein bands of fragment D and E respectively, indicating that the digestion had run its full course. The rest of the gels, representing digests of the various cross-linked samples, were identical to the control with no evidence of high molecular weight bands, thus indicating that no cross-linking had occured between the core domains. This therefore suggests that the D and E domains are not involved in the conformational changes which occur in the different buffers.

Section (4) : Calcium Studies

Various investigators have shown that fibrinogen from several species has both high and low affinity binding sites for calcium (Marguerie et al, 1977; Van Ruijven-Vermeer et al, 1978; Kemp et al, 1983;) The importance of tightly bound calcium ions ($K_d \simeq 10^{-6}M$) to fibrinogen is well established, however the role of the larger number of low affinity sites ($K_d \simeq 10^{-3}M$) is less clear.

All results from the previous section suggest that calcium ions have a direct effect on the conformation of fibrinogen, rendering the latter more open. In addition, the results from both techniques correlate with each other.

The aim of this and subsequent experiments was to investigate fibrinogen conformation at a range of calcium concentrations around the physiological level. This was done via photosensitized cross-linking and radioactive photosensitized labelling.

Photosensitized Cross-Linking

Fibrinogen samples (concentration, 2.94 nM) were cross-linked for 30 seconds in 0.05M Tris-HCl, 0.05M NaCl, pH 7.5, containing calcium concentrations ranging from 0 to 13.5 mM. As seen previously, the high affinity sites are assumed to be

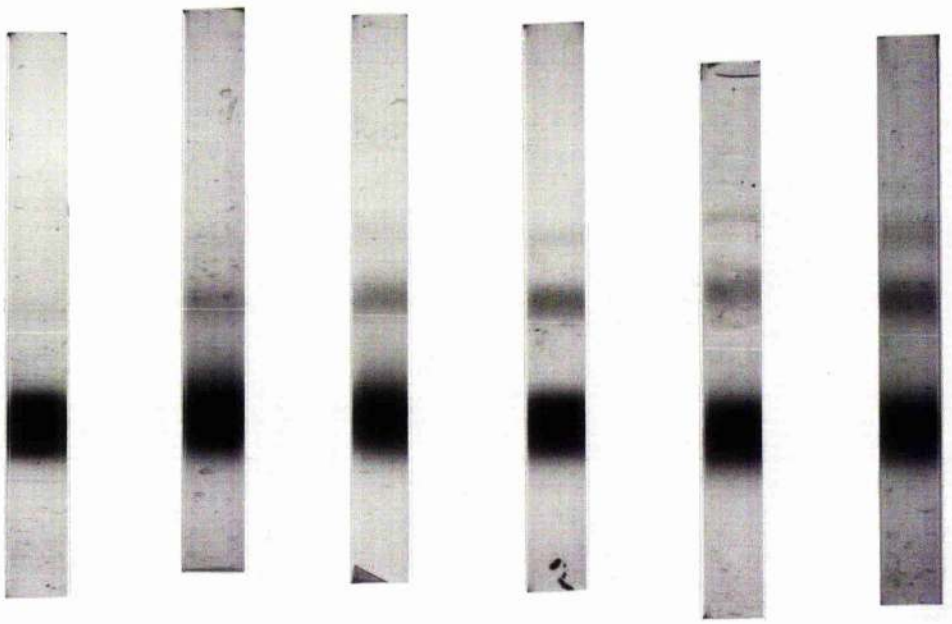
occupied at the "zero" calcium concentration used in these experiments.

3% and 5% SDS-polyacrylamide gels were then run for the non-reduced and reduced samples respectively.

The proportion of intermolecular cross-linking (Figure 3-16 (a)) was found to increase with an increased calcium concentration. This is further illustrated in Figure 3-17; here the 3% polyacrylamide gels were scanned and the percentage variation of polymer/monomer plotted against calcium concentration. This evidence confirms earlier results and shows that the fibrinogen molecule adopts a more open conformation as the calcium concentration is increased.

Analysis of the reduced cross-linked samples (Figure 3-16 (b)) revealed that the proportion of cross-linked Aα-chain increased as the calcium concentration was increased, particulary at calcium concentrations where there is more inter-molecular cross-linking. On the other hand, the Bβ and γ-chains are relatively unaffected. This suggests that the increase in the proportion of intermolecular cross-linking occurs mainly through the C-terminal portions of the Aα-chains.

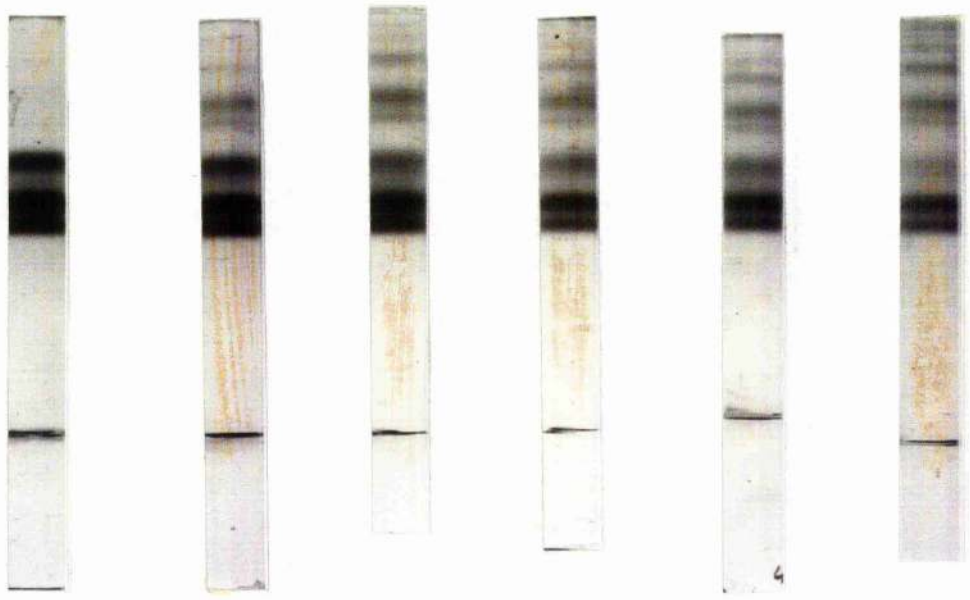
The cross-linked fibrinogen samples plus a control of uncross-linked fibrinogen were then digested using plasmin, according to the method described in Chapter Two. This was done



Calcium concentration (mM) ⟶

c = *control (ie. no cross-linking)*

Figure 3-16(a): SDS-polyacrylamide gels (3% acrylamide) of fibrinogen cross-linked at various calcium concentrations.



c = control (ie. no cross-linking)

Figure 3-16(b): SDS-polyacrylamide gels (5% acrylamide) of fibrinogen cross-linked at various calcium concentrations, then reduced.

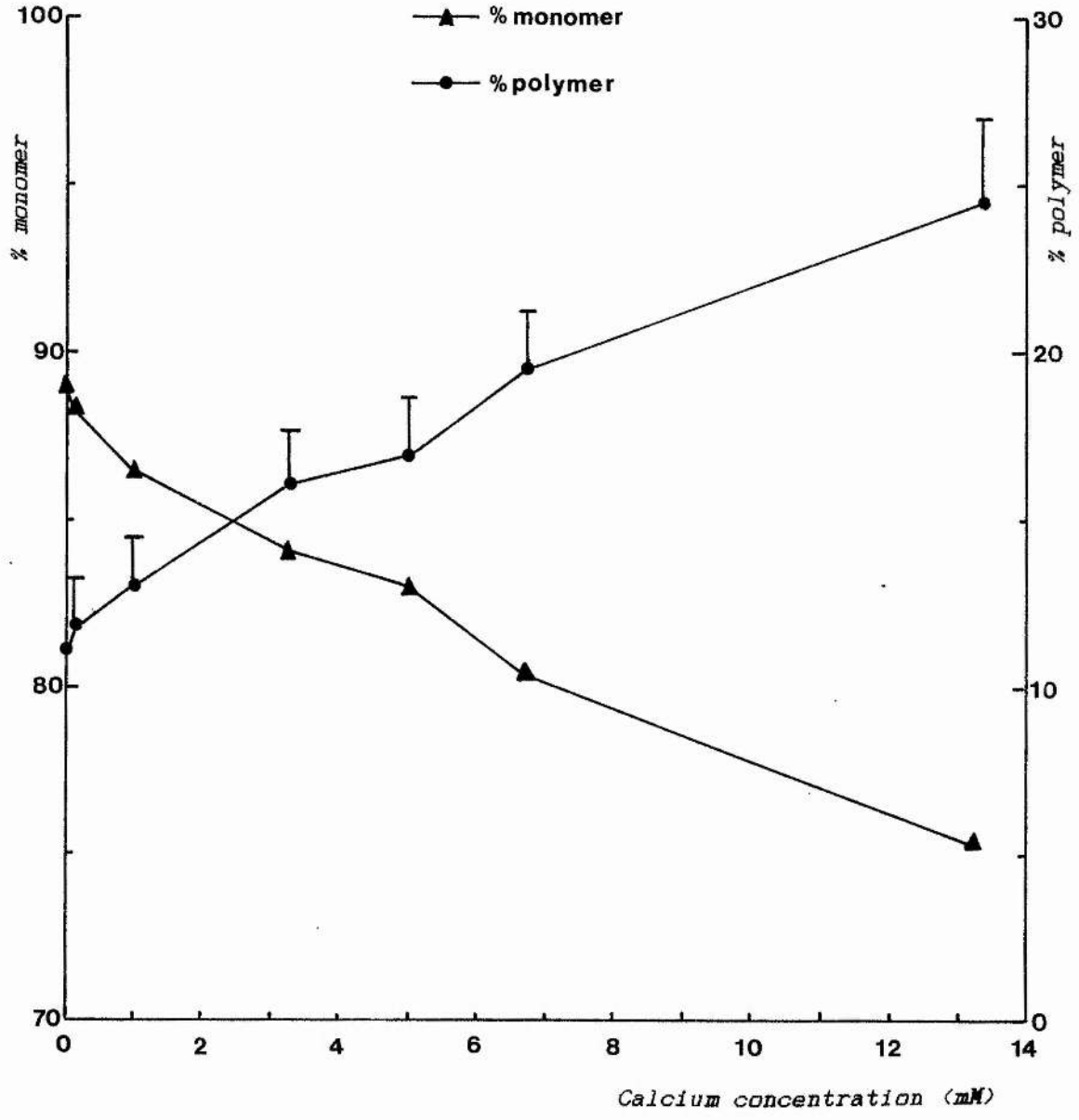


Figure 3-17: Intermolecular cross-linking of fibrinogen as a function of calcium concentration.

The points represent the mean of two experiments.

with the aim of investigating whether or not the D and E domains are involved in the calcium-induced conformational change of fibrinogen.

5% SDS-polyacrylamide gels of the non-reduced samples were run after a digestion time of six hours. Analysis of these gels showed that the digest products of the various cross-linked samples were the same as those on the control gel. There was no evidence of high molecular weight bands on any of the gels indicating that the D and E domains do not interact during the conformational changes which occur as a result of the influence of calcium.

Photosensitized Radioactive Labelling

These experiments were carried out with the aim of investigating whether this technique would also detect the calcium-induced conformational change of the protein demonstrated in previous experiments.

Fibrinogen (final concentration, 1.32nM) was surface-labelled for 20 seconds, at a range of calcium concentrations, using the method described earlier. The radioactivity of the solubilized protein bands for gels of fibrinogen samples was measured by scintillation counting.

Results of the experiment can be seen in Figure 3-18. The latter shows that as the concentration of calcium increases, the amount of label incorporated into the molecule also increases. This corresponds to an increase in the surface area of the molecule exposed to solvent. These results correlate with those from previous experiments and reaffirm the suggestion that fibrinogen adopts a more open conformation in the presence of calcium.

The change in conformation appears to occur in two phases, with an increase in surface area accompanying an increase in calcium concentration upto 2mM and a further opening out of the molecule occuring above approximately 3.5mM $CaCl_2$. The significance of the decrease in incorporation which occurs between the calcium concentrations of 2-3.5mM will be discussed later. It should also be noted that this biphasic effect occurs over a range encompassing both the physiological calcium concentration as well as the dissociation constant of the low affinity calcium binding sites.

The photolabelled fibrinogen samples were then digested with plasmin. The aim of this experiment was to measure incorporation of radioactivity into the two fibrinogen core domains, D and E, respectively.

The labelling pattern seen in Figure 3-19 was obtained. It was found that the labelling of both major domains is affected,

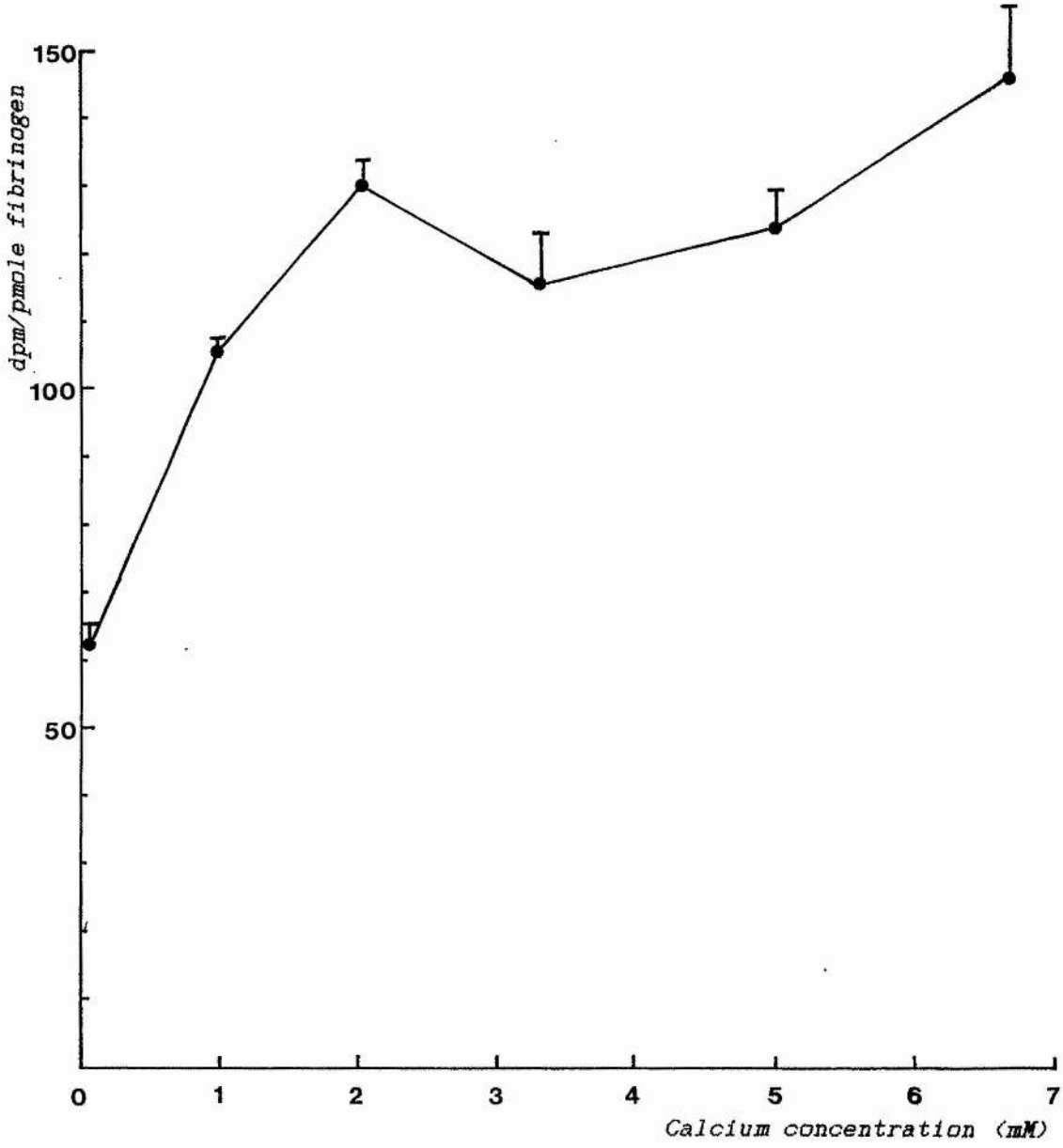


Figure 3-18: The surface labelling of fibrinogen as a function of calcium concentration.

The points represent the mean of two experiments.

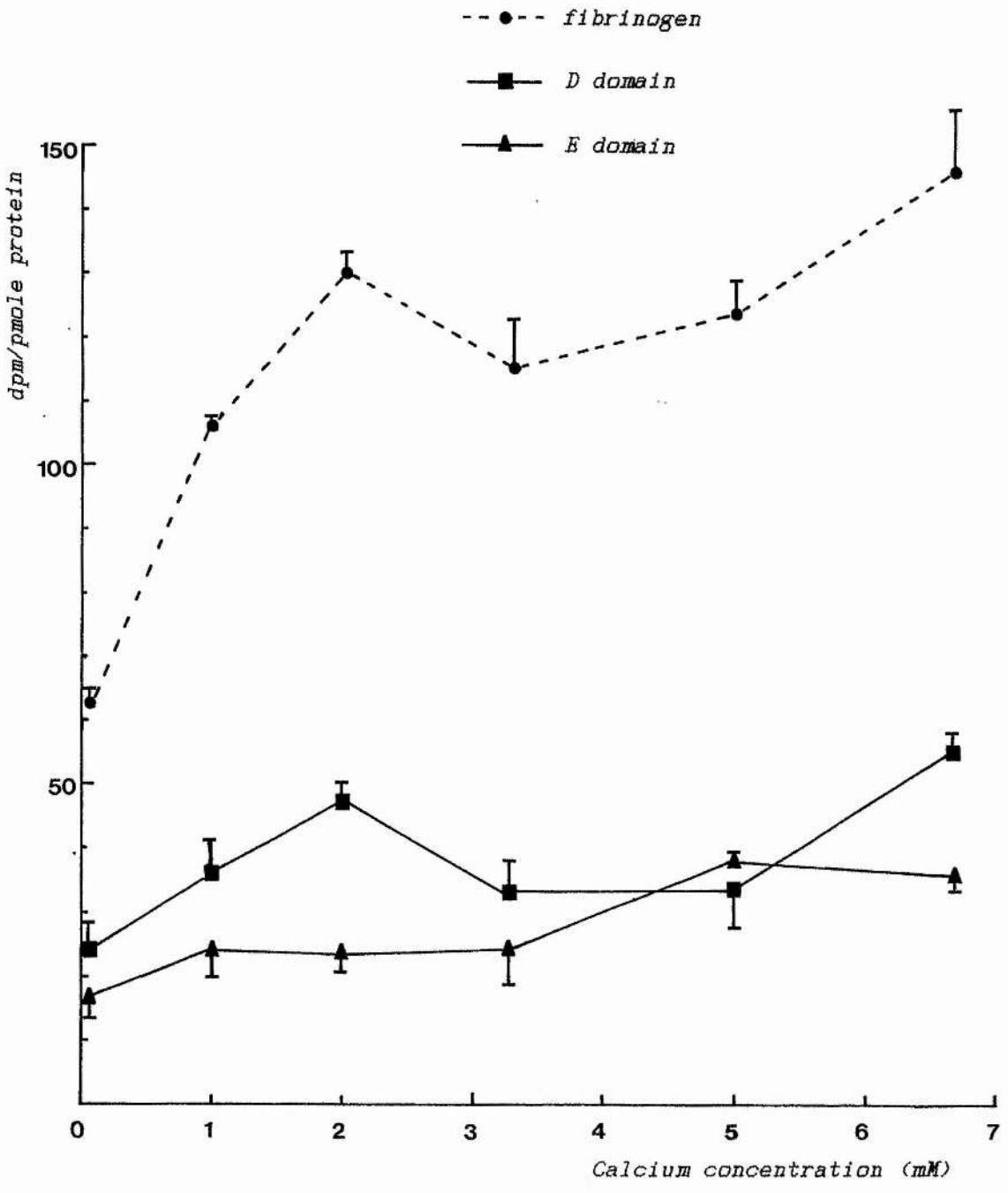


Figure 3-19: Incorporation of label into the D and E domains as a function of calcium concentration.

The points represent the mean of four experiments.

but not identically. Incorporation into the D domains is influenced in both phases whereas incorporation into the E domain is mainly affected in the second phase.

Incorporation into the E domain does not vary much initially but increases as the calcium concentration is raised. This implies that the central or E domain of the molecule does not constitute a major part of its surface at the lower end of the calcium concentration range examined. As the concentration of calcium is increased, however, the E domain becomes more exposed and incorporation of label increases.

If the percentage of Aα-chain that is not taken up by cross-linking (as the calcium concentration is increased) is compared to the degree of labelling incorporated into the E domain (see Figure 3-20), it can be seen that the two are interrelated. Greater surface labelling of the E domain was reflected by the disappearance of uncross-linked Aα-chains, both effects increasing as the calcium concentration was raised. This therefore suggests that the increased incorporation into the E domain is due to the uncovering of this region rather than the effect of a conformational change occurring within the E domain.

The incorporation into the D domain is affected in both phases. These changes in the surface labelling of the D domains could be due either to conformational changes within these domains or to a calcium-induced exposure of these regions by

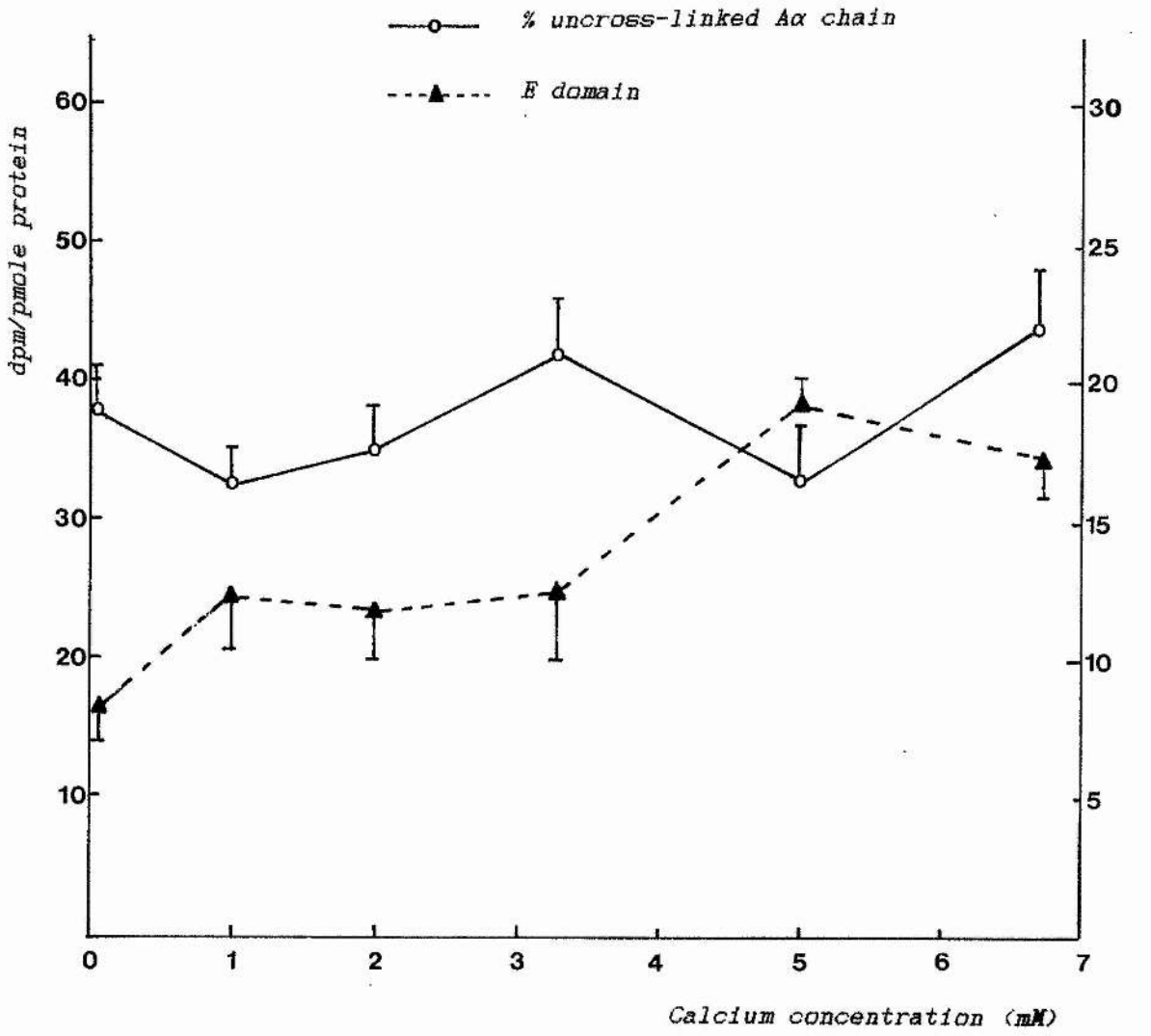


Figure 3-20: Relationship between E accessibility (ie. label in E domain; dpm/pmole protein) and % uncross-linked Aα chain.

The points represent the mean of two experiments.

some other part of the molecule. In order to distinguish between these possibilities, in the next experiment the effect of calcium on surface labelling of fragment D was compared to similar results for the D domain. A similar experiment was also carried out for fragment E and the E domain.

At this stage it is also interesting to note that when the sum of the incorporation into the core domains is plotted (Figure 3-21) the resulting trend compares very well to that of incorporation into whole fibrinogen. Thus, as is expected, the labelling of the core domains is directly reflected in that of the whole molecule.

In the next experiment, fragment D and fragment E (concentrations 2.64nM and 1.32nM respectively) were each labelled at a range of calcium concentrations. This was done in order to determine whether calcium-induced changes in the incorporation of the D and E domains are due to either exposure or conformational changes within these regions.

The results in Figure 3-22 suggest that in the first phase there are changes in the accessibility of the D domain. This area of the molecule is first made available, but is hidden again as the calcium concentration rises. However, in the second phase, although a sharp increase in the incorporation of the fragment occurs at approximately 3.5mM $CaCl_2$, this effect is somehow masked in the D domain. This suggests that despite the

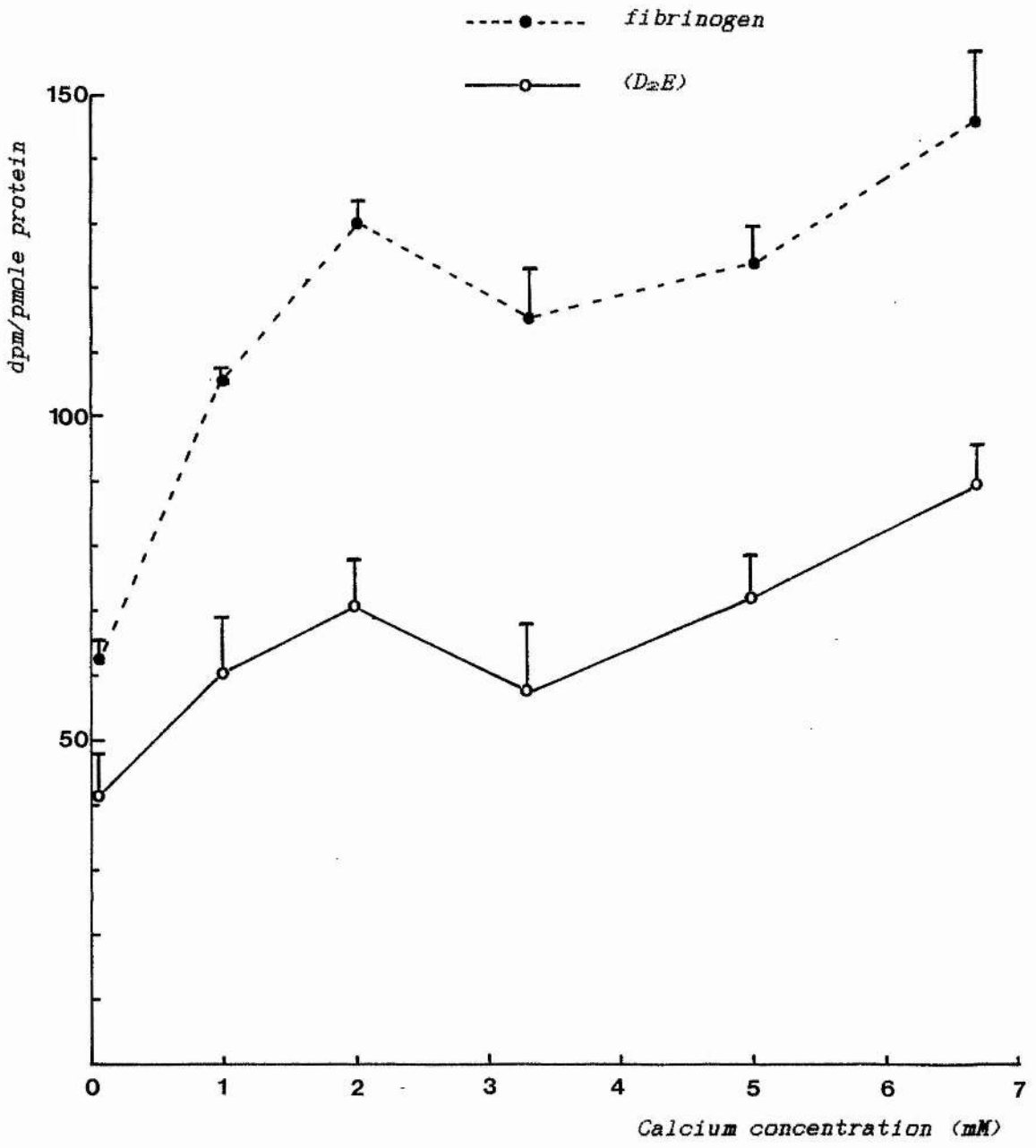


Figure 3-21: Graph of the incorporation into fibrinogen and the sum of the incorporation into the core domains, as a function of calcium concentration.

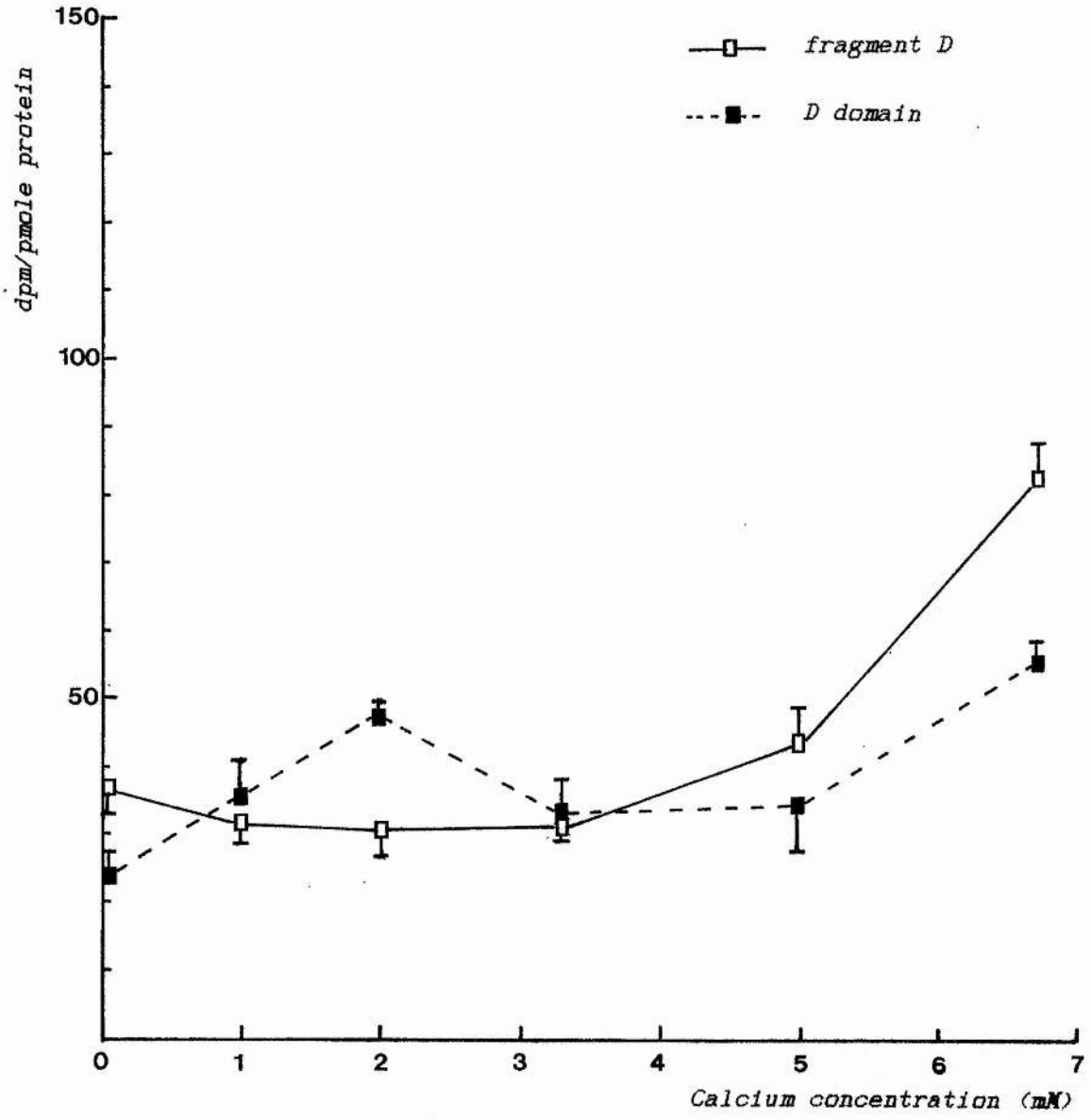


Figure 3-22: Comparison of the incorporation of label into the D domain and fragment D as a function of calcium concentration.

The points represent the mean of three experiments.

increase in the incorporation of the fragment which occurs at this stage and upto a calcium concentration of 5mM, the D domain is still occluded by some other part of the molecule. The results thus suggest that this increase in the incorporation of the fragment at 3.5mM $CaCl_2$ is due to a conformational change which renders this part of the molecule more open and thus more accessible to the radioactive label. The effect of this conformational change is somehow masked in the whole molecule.

An experiment to determine incorporation into the Aα-chains over a range of calcium concentrations was carried out. The latter was also calculated theoretically by subtracting the sum of the incorporation into the core domains (ie. D_2E) from that of incorporation into whole fibrinogen. The result should approximate to the contribution from the C-terminal two-thirds of the Aα-chains. As can be seen from Figure 3-23, the experimental and theoretically derived values are in good agreement.

Definite similarities are seen when comparing the results for the D domain with those for incorporation into the Aα-chain (see Figure 3-24). Incorporation into the Aα-chain parallels that of the D domain. This would suggest that the Aα-chains may, at least in part, be responsible for the occlusion of the D domains.

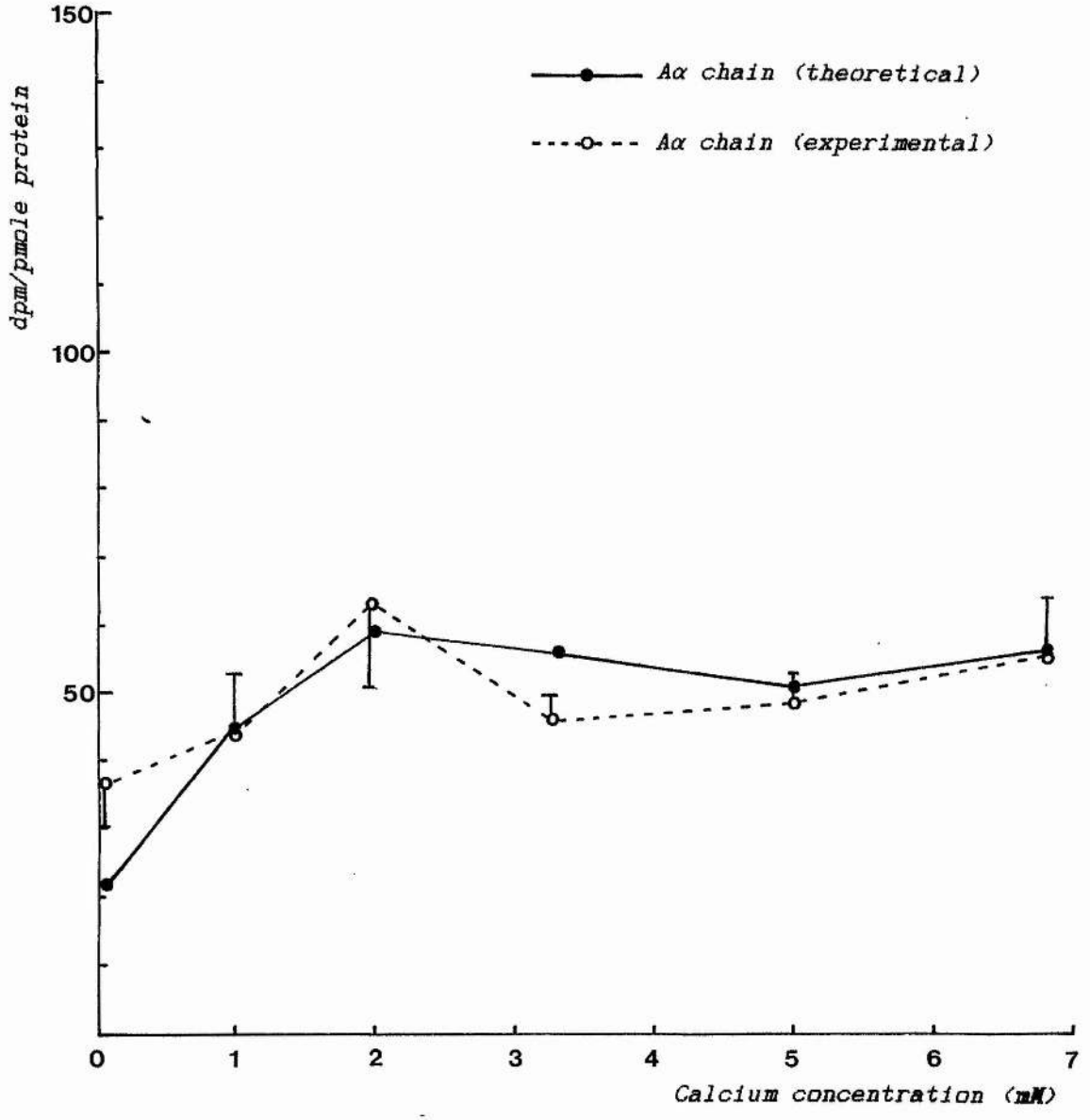


Figure 3-23: Graph of incorporation of label into the Aα chains as a function of calcium concentration.

The points represent the mean of two experiments.

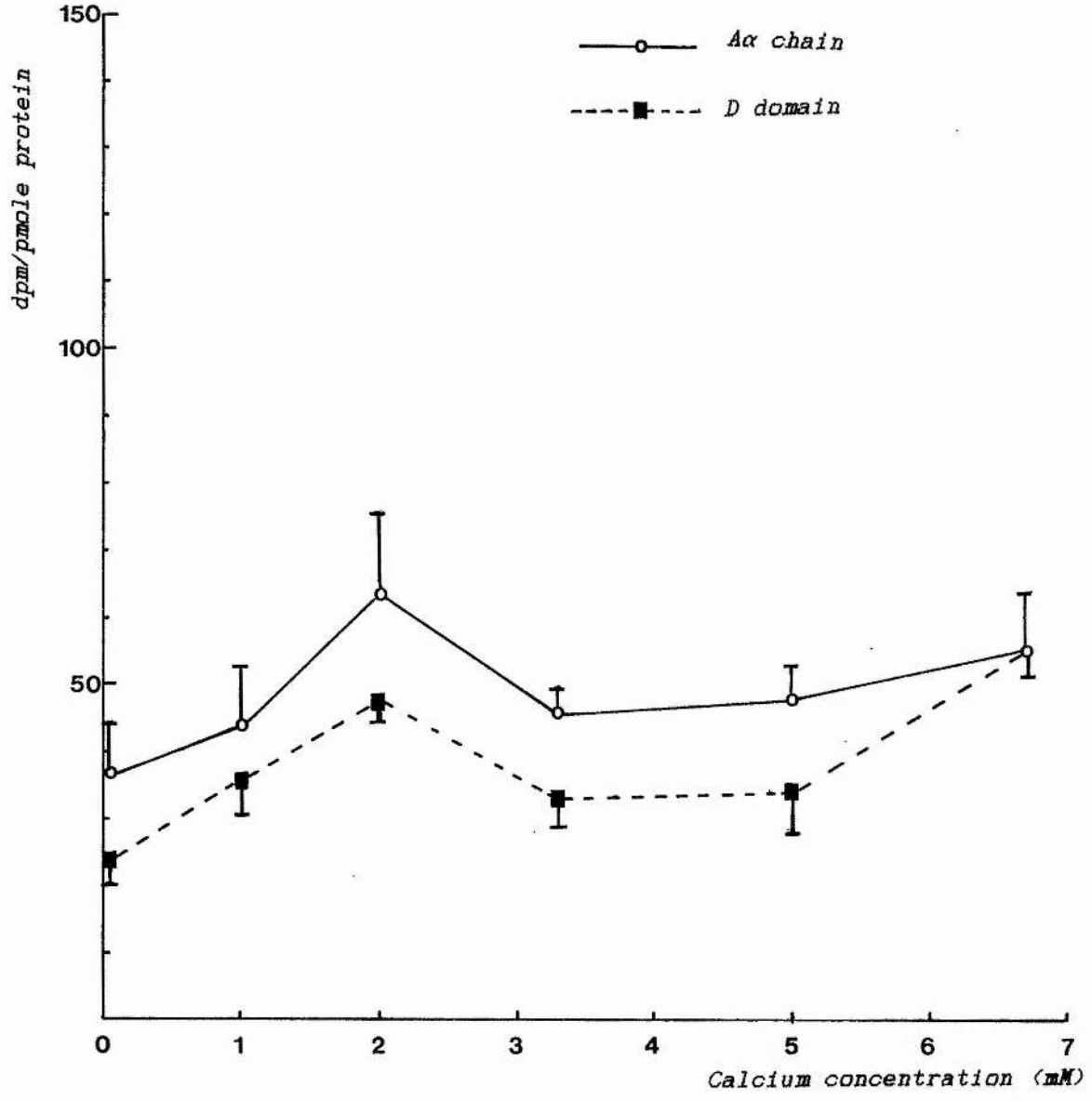


Figure 3-24: Comparison of the incorporation of label into the D domain and the Aα chains as a function of calcium concentration.

The points represent the mean of two experiments.

The incorporation into the E fragment in comparison with the E domain can be seen in Figure 3-25. It was found that the overall incorporation into fragment E is much higher when compared to that of the domain. This suggests that fragment E has a very different conformation to its domainal form and thus the two cannot be compared directly. This was also seen when the experiment was performed in different buffer systems (Table 3-ix). The high incorporation into the E fragment could be due to the increase in the surface area and stearic freedom of the fragment, compared to the rigid and tightly held domainal region found in the whole molecule. However, if one simply considers the trend of incorporation (Figure 3-25) it can be seen that, in general, the fragment tends to incorporate progressively more label as the calcium concentration is increased. However, this tendency is suppressed in the E domain due to occlusion by some other part of the molecule.

The Effect of Magnesium and Sodium Chloride on Fibrinogen

According to Marguerie at al (1977) fibrinogen has several binding sites of low affinity which, in the presence of 10^{-2}M $MgCl_2$ are eliminated, suggesting that these binding sites are not specific and are due to weak interactions.

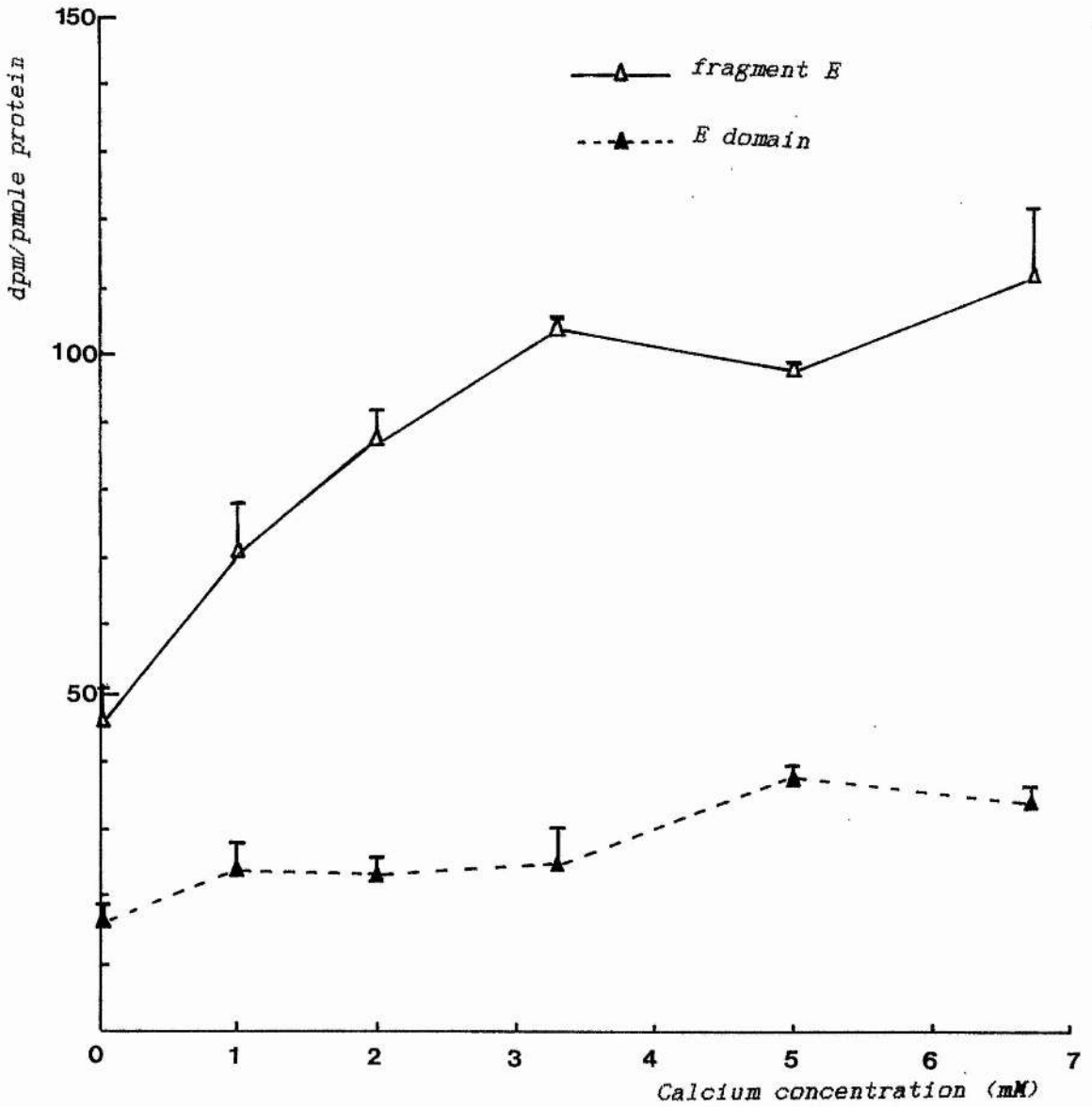


Figure 3-25: Comparison of the incorporation of label into the E domain and fragment E as a function of calcium concentration.

The points represent the mean of two experiments.

Table 3-(ix): Incorporation of Radioactive Label Into The E Domain and Fragment E.

BUFFER	E DOMAIN	FRAGMENT E
0.05M Tris-HCl, 2mM $CaCl_2$, pH = 7.5	23.8	65
0.13M NaCl, 6.7mM sodium phosphate, pH = 7.5	117.4	187.9

Results are expressed as dpm/pmole protein and represent the mean of six experiments.
In all cases the standard error was $\leq \pm 7.5\%$, and the significant difference, p, was ≤ 0.001.

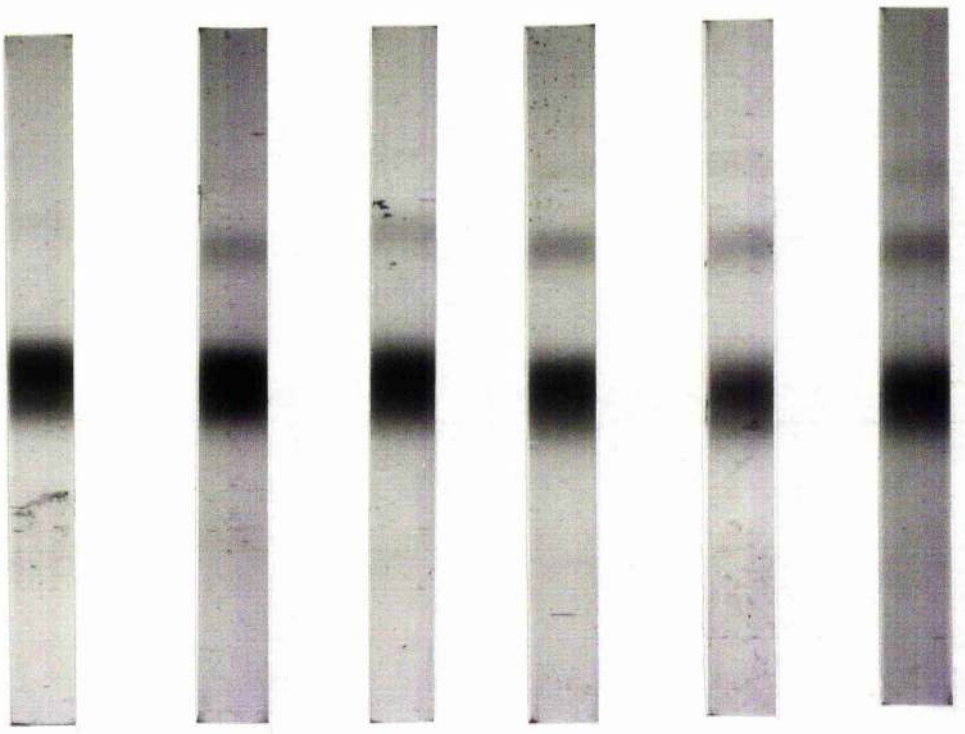
The following experiments were done with the aim of investigating whether calcium is specifically needed for the conformational changes seen to occur in previous experiments.

Photosensitized Cross-Linking

Fibrinogen samples were cross-linked in 0.05M Tris-HCl, 0.05M NaCl, pH 7.5, containing magnesium chloride concentrations ranging from 0 to 13.5 mM. Samples used for cross-linking at zero magnesium were dialysed seperately from those cross-linked in magnesium. Fibrinogen used in the latter experiments was first dialysed into the above buffer containing 0.05mM $MgCl_2$. It should also be noted that the high affinity calcium binding sites were considered to be occupied at the zero magnesium concentration used in these experiments.

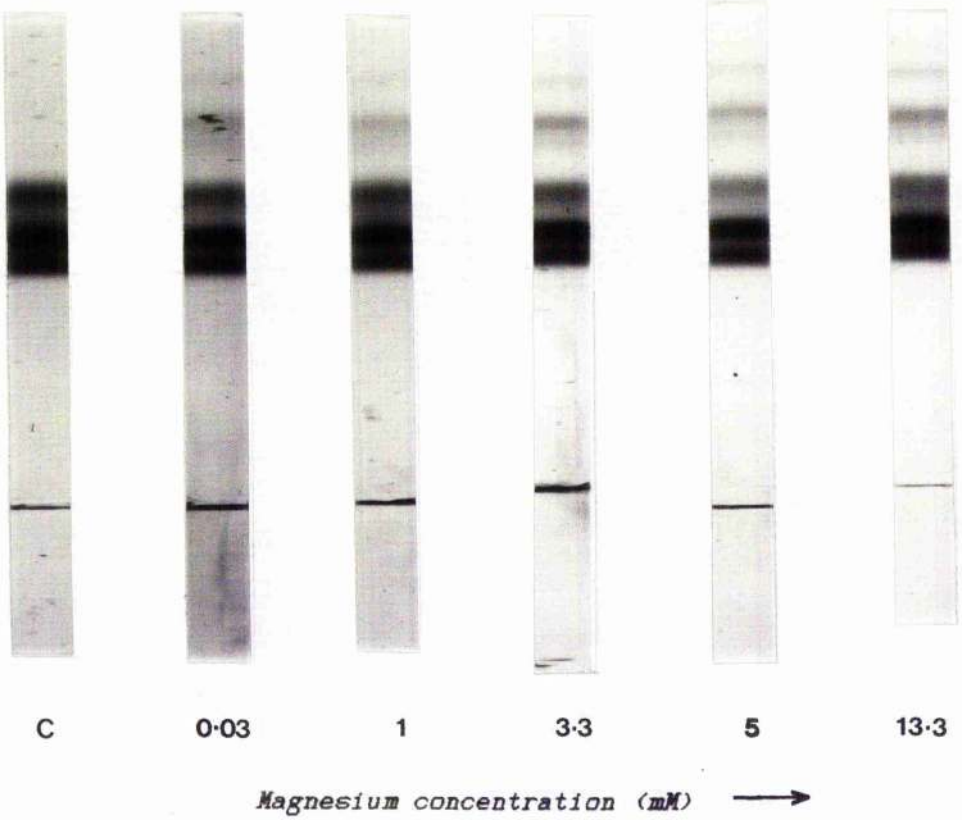
SDS-polyacrylamide gels of non-reduced (3% acrylamide) and reduced (5% acrylamide) cross-linked samples can be seen in Figures 3-26 (a) and (b) respectively.

The 3% gels show that to some extent the proportion of intermolecular cross-linking does increase with magnesium and therefore the latter does have some effect on the unfolding of the molecule. However, this effect is markedly less than that which occurs in calcium. The amount of polymer produced in the presence of magnesium starts to level off at 5mM whereas it is still on the increase in calcium (see Figure 3-27).



c = control (ie. no cross-linking)

Figure 3-26(a): SDS-polyacrylamide gels (3% acrylamide) of fibrinogen cross-linked at various magnesium concentrations.



c = control (ie. no cross-linking)

Figure 3-26(b): SDS-polyacrylamide gels (5% acrylamide) of fibrinogen cross-linked at various magnesium concentrations, then reduced.

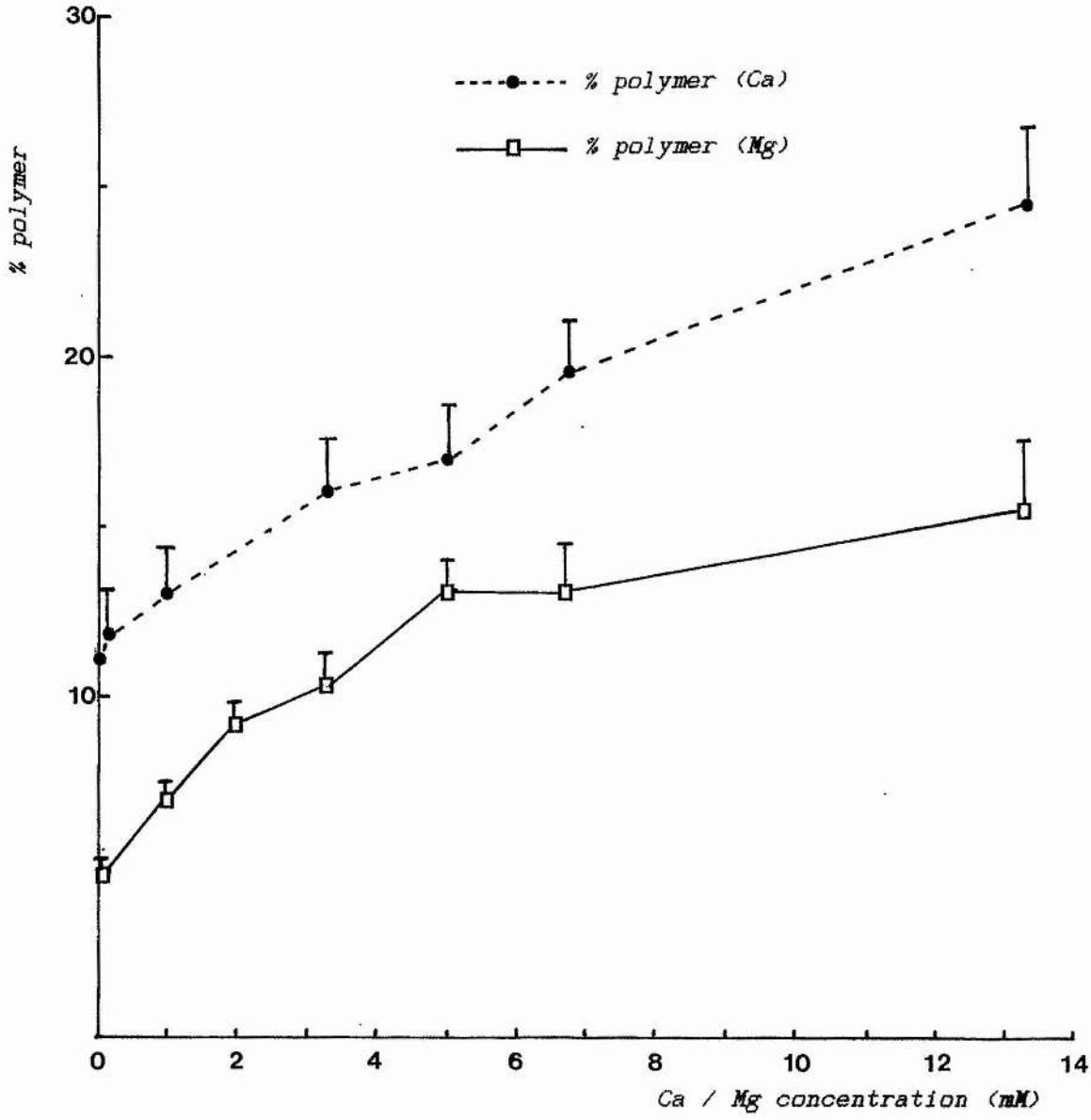


Figure 3-27: Comparison of the effect of calcium / magnesium on intermolecular cross-linking.

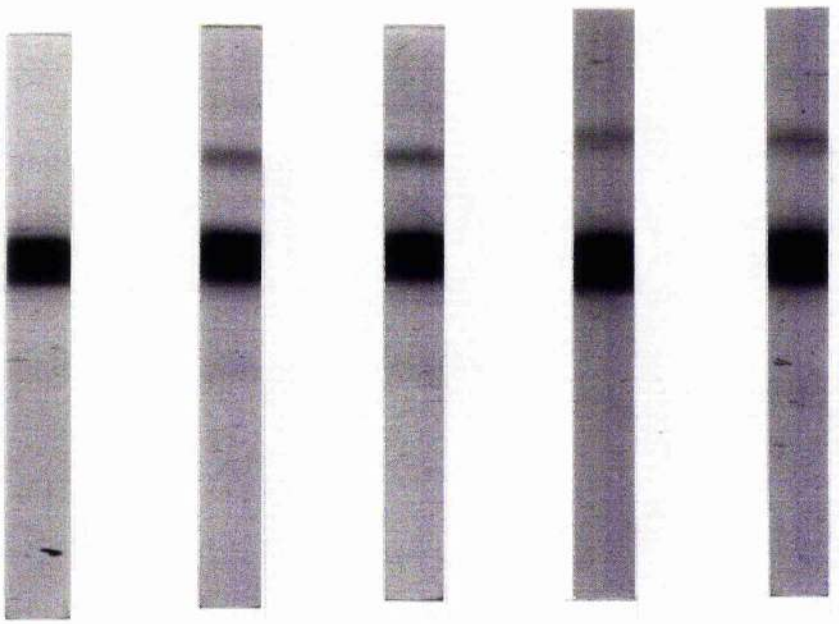
The points represent the mean of two experiments.

This is also reflected in the 5% gels (see Figure 3-26(b)) where the resulting number of cross-linked bands are less in magnesium compared to those for the calcium experiment (see Figure 3-16(b)). It should also be noted that the Aα-chain is the one decreasing in intensity compared to the Bβ and γ-chains which remain relatively unaffected. This suggests that the Aα-chain is still the first subunit to be taken up in cross-linking.

The aim of the sodium chloride experiments was to see if any other ion, such as sodium, has an effect on the conformation of fibrinogen.

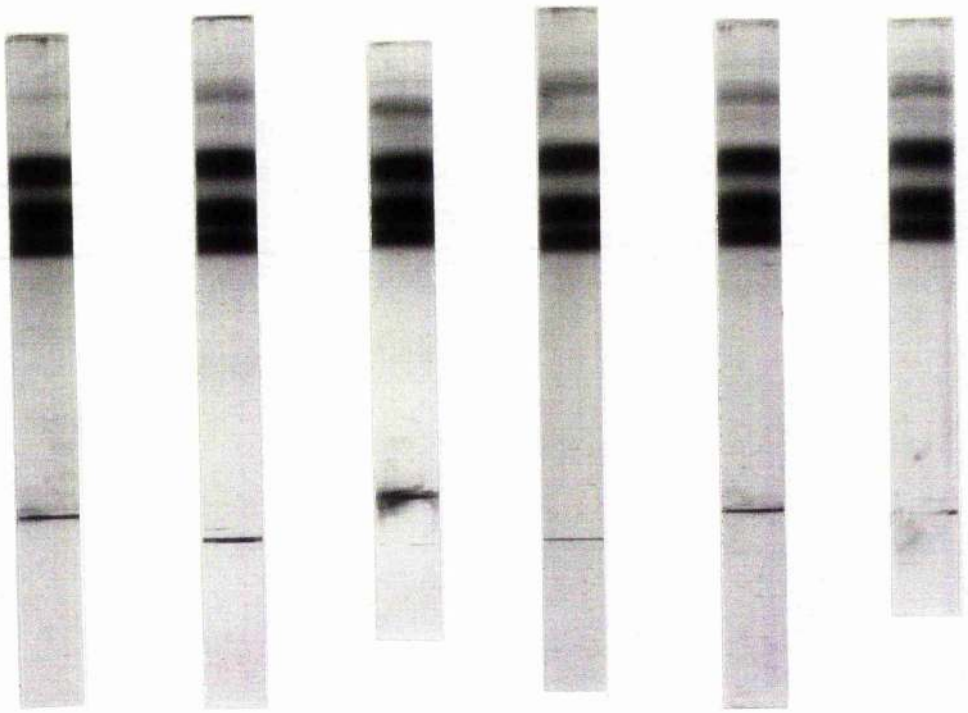
Fibrinogen was cross-linked in 0.05M Tris-HCl, containing sodium chloride concentrations ranging from 0 to 40mM. (Ionic strength differences were compensated for). Gels of non-reduced and reduced cross-linked samples were run on 3% and 5% acrylamide gels respectively (see Figures 3-28 (a) and (b)).

Intermolecular cross-linking patterns on the 3% gels showed that although dimer formation did result, not only was this the only cross-link band seen, but the amount of dimer formed was minimal compared to the amount of polymer formed in the calcium experiment. The maximum amount of dimer present on the gel representing the highest sodium chloride concentration was 5.5%.



c = control (ie. no cross-linking)

Figure 3-28(a): SDS-polyacrylamide gels (3% acrylamide) of fibrinogen cross-linked at various sodium chloride concentrations.



c = control (ie. no cross-linking)

Figure 3-28(b): SDS-polyacrylamide gels (5% acrylamide) of fibrinogen cross-linked at various sodium chloride concentrations, then reduced.

The cross-linking patterns on the 5% gels showed that no increase in the amount of cross-linked bands occurred over the range of sodium chloride concentrations. The extent of cross-linking is also minimal and there is no appreciable decrease in the intensity of either of the Aα, Bβ or γ-bands over the range of sodium chloride concentrations tested.

Radioactive Photosensitized Labelling

The aim of these experiments was to investigate the effect of magnesium and sodium chloride on the radioactive photosensitized labelling pattern of fibrinogen. The results obtained were then compared to those resulting from calcium studies.

Fibrinogen was surface labelled in 0.05M Tris-HCl, 0.05M NaCl, pH 7.5, containing magnesium chloride concentrations ranging from 0 to 13.5mM. The result of this experiment (Figure 3-29) shows that although magnesium has a similar effect to calcium in that the molecule adopts a more open conformation, higher magnesium concentrations are required to produce equivalent effects.

Fibrinogen was then photolabelled in sodium chloride over a similar range of ionic strengths. The results (see Figure 3-30) show that sodium chloride has no effect in rendering the conformation of fibrinogen more open.

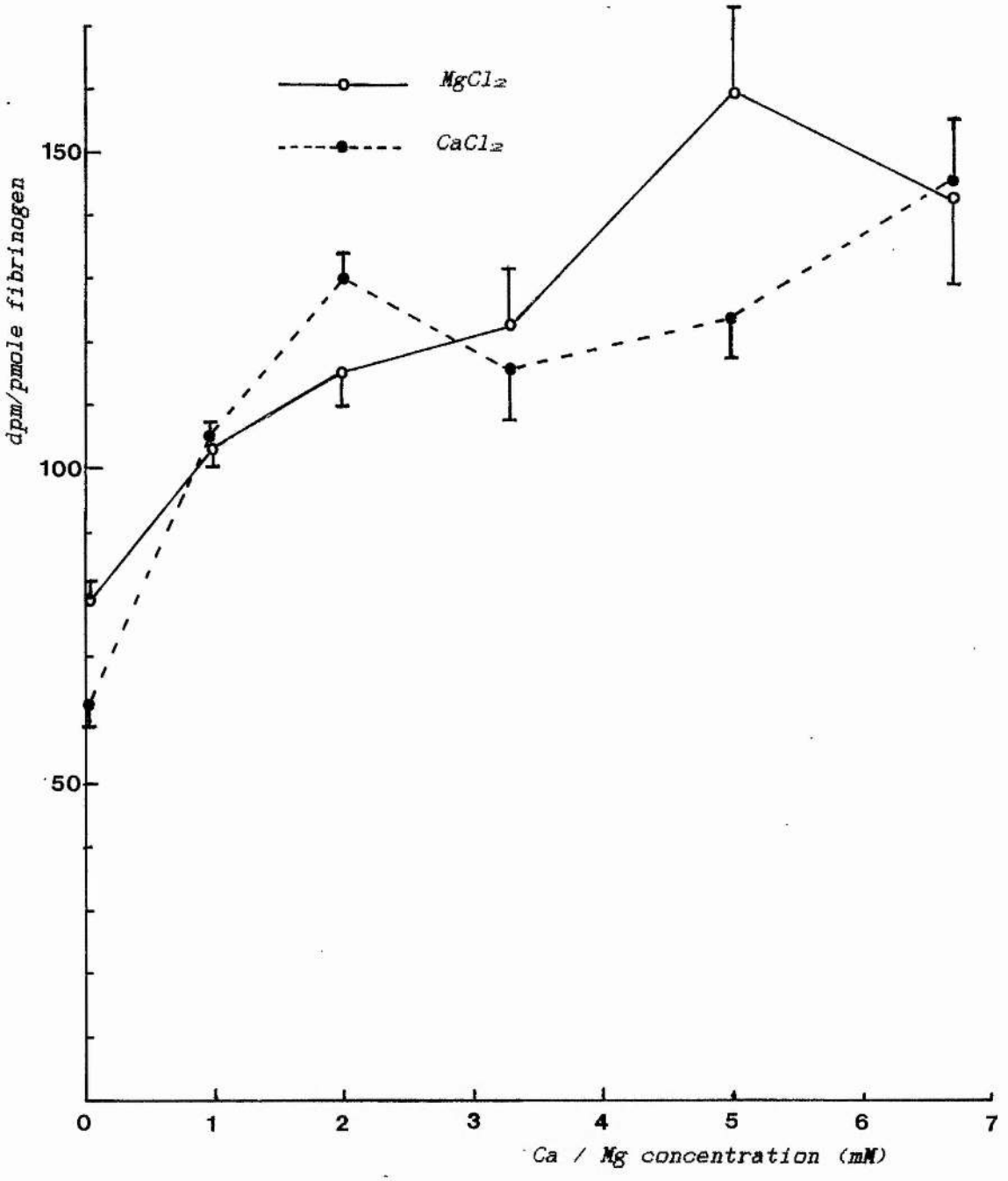


Figure 3-29: Comparison of the effect of calcium / magnesium on the photosensitized radioactive labelling of fibrinogen.

The points represent the mean of two experiments.

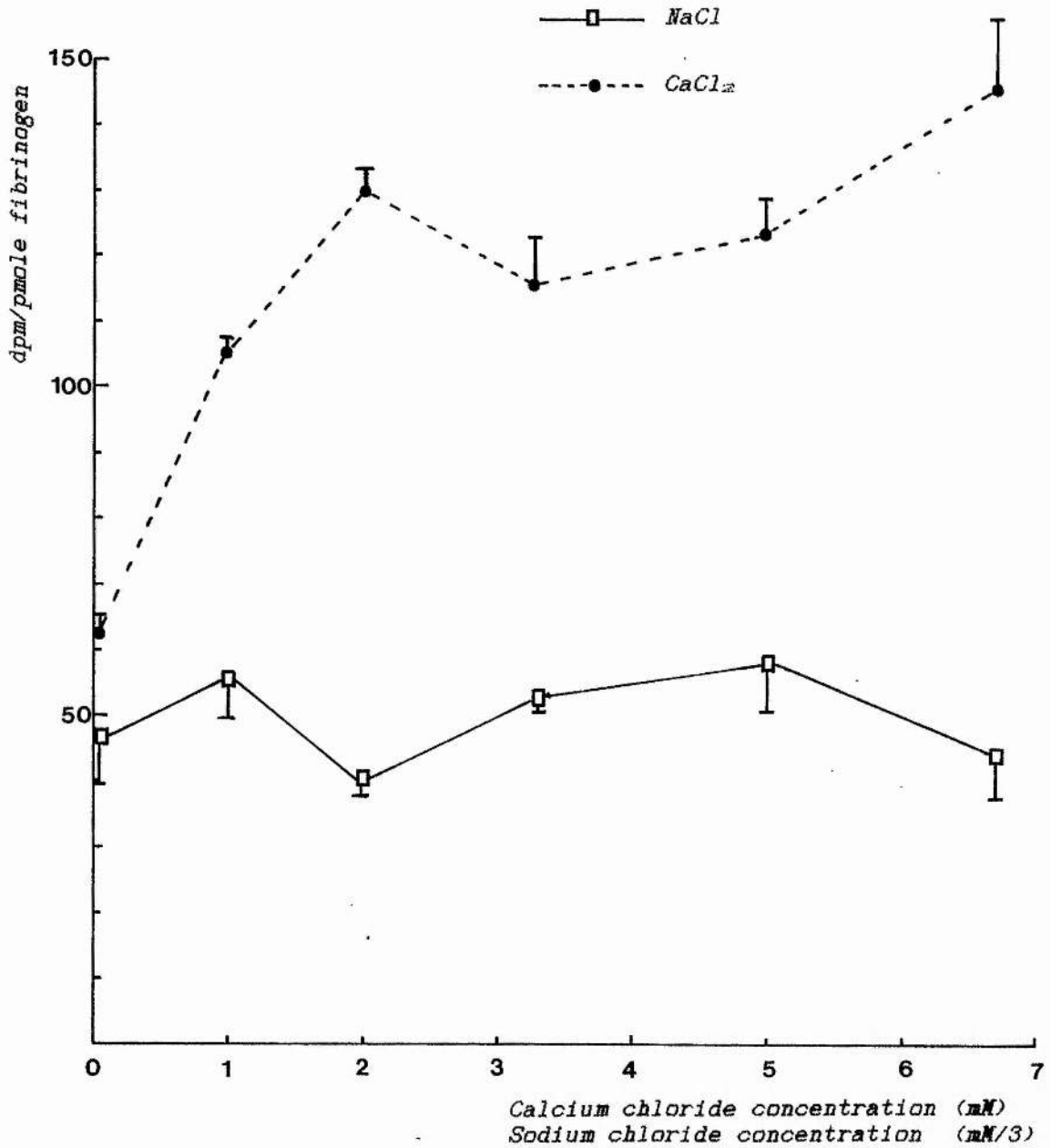


Figure 3-30: Comparison of the effect of calcium / sodium chloride on the photosensitized radioactive labelling of fibrinogen.

The points represent the mean of two experiments.

CHAPTER FOUR

DISCUSSION

The aim of this work was to develop and assess the two techniques of photosensitized radioactive surface labelling and photosensitized cross-linking with a view to examining the conformation of fibrinogen in its native state and under different solvent conditions, with particular reference to the influence of calcium.

Prior to carrying out the above studies, the primary concern was to isolate a stable fibrinogen preparation from human plasma. The two main priorities concerning this step were that the fibrinogen had to be of good quality (ie. with a high degree of intactness) and free of contaminating proteins. These two points are very important in view of the fact that this study is primarily concerned with the shape of the molecule.

Normal human plasma contains fibrinogen of different molecular weights. Mills and Karpatkin (1971) suggested that this heterogeneity was due to the proteolytic action of thrombin on the molecule. On the other hand, Mosesson et al (1974) attributed this heterogeneity to in vivo degradation of the Aα-chain by plasmin, whilst Semeraro et al (1977) proposed that the majority of Aα-chain heterogeneity is the result of storage of plasma which leads to in vitro cleavage of this subunit. Phillips (1981) developed a method to isolate and separate two molecular sizes of fibrinogen, FI (high molecular weight form) and FII (lower molecular weight form), which had been found by

Lipinska et al (1974). The difference between these two forms lies in their respective Aα-chain content. SDS-PAGE of FII showed that this contains only one intact Aα-chain. On the other hand Holm and Godal (1984) used fibrin to exclude non-clottable proteins from their preparations and demonstrated that normal plasma fibrinogen contains three major fibrinogen fractions which differ in molecular weight. The HMW fraction (molecular weight 340,000) contains intact Aα-chains while the molecular weight of fractions LMW and LMW' is reduced to 305,000 and 270,000 respectively due to proteolysis of the C-terminal parts of one (LMW), or both (LMW'), Aα-chains (Holm et al, 1985a).

The most important conclusion that can be drawn from these studies is that the Aα-chain represents a highly labile portion of the molecule. Variations in this subunit's molecular weight probably represent a combination of in vivo and in vitro cleavage; thus a good course of action for purifying fibrinogen would be to adopt as rapid a procedure as possible so as to minimize in vitro cleavage. Plasmin contamination of the fibrinogen preparation must also be avoided.

In view of the fact that fibrinogen binds calcium and that it is resistant to denaturation (Ly and Godal, 1973) and digestion (Marguerie, 1977; Haverkate and Timan, 1977;) in the presence of calcium, and also because a major part of this study concerns the influence of calcium on the structure and properties of fibrinogen, a purification procedure where calcium

is included was selected. Due to this inclusion, however, a preparation free of prothrombin and factor XIII is required. This is so since calcium ions accelerate activation of prothrombin to thrombin and the latter converts fibrinogen to fibrin. Thrombin and calcium ions are also involved in the activation of the proenzyme, factor XIII, to its active form, factor XIIIa, by the removal of a short peptide (Lorand and Konishi, 1964). The function of factor XIIIa is to cross-link and stabilize fibrin covalently via the formation of isopeptide bonds in the final step of fibrin formation and this stage also requires the presence of calcium ions. Thus for these reasons calcium should be left out of buffers used until prothrombin is removed from the system. Several proteins, including prothrombin, tend to co-precipitate with fibrinogen and therefore specific techniques must be included in the purification of the molecule in order to remove such contaminants quickly and efficiently.

In this study, purification was carried out by a method adapted from that of Lawrie et al (1979). By means of this method both fibrinogen and plasminogen (and consequently the core fragments D and E) can be purified from the same plasma sample. Plasminogen is removed from plasma by passing the latter over a lysine-sepharose column; here plasminogen (or plasmin) attaches to the lysine groups on the column, in preparation to be displaced at a later stage by ϵ-amino caproic acid. Prothrombin is removed from the plasminogen-free plasma by the

addition of an aluminium hydroxide suspension onto which it is absorbed.

Various procedures may be used to precipitate fibrinogen out of solution, including salt or organic solvent fractionation. The latter method is based upon differences in the solubility of proteins in aqueous solutions of such organic solvents as ethanol, acetone, butanol or, as Kekwick et al (1955) used, ether. (The latter method is the one used by authors Pouit et al (1972), Hudry-Clergeon et al (1975), and Marguerie and Stuhrmann (1976). - (See Chapter One, Table 1-(iv), and later in this chapter). However such solvents may cause denaturation which results in changes in the molecule's conformation. Consequently in this method salt fractionation, involving the use of ammonium sulphate, was used to precipitate out fibrinogen.

After dialysis the suspended precipitate is applied to a DEAE-cellulose column. It is important to note that only at this stage is calcium introduced into the buffer system. Three peaks are eluted off the column using buffers made progressively more concentrated in NaCl. Peak I fibrinogen has a lower negative charge compared to the peak II type and thus elutes first. As judged by gel electrophoresis, peak I fibrinogen shows little degradation compared to that contained in peak II and constitutes the more intact fraction. Factor XIII is also eluted in peak II. The third peak (III) contains fibronectin

which is known to associate with fibrinogen. Thus in this case ion exchange chromatography serves two main purposes: (i) the seperation of fibrinogen types on the basis of their intactness, and (ii) the removal of remaining co-precipitating protein contaminants. In the studies to follow, only peak I fibrinogen was used. The method of purification described here allows the isolation of high quality fibrinogen in under eight hours.

The core fragments D and E were prepared from the purified fibrinogen using a by-product of the above method, ie. plasmin(ogen), according to the procedure described in Chapter Two. The digestion was done in the presence of calcium (1mM), as the result of a report by Haverkate and Timan (1977) who found that D(cate) (ie. the final fragment D product with calcium present) is protected from further plasmin attack in the presence of calcium. (N.B. As stated earlier, from here on this fragment will simply be referred to as fragment D). This system also approximates physiological conditions with respect to the calcium concentration. The digest mixture was then applied to a DEAE-cellulose column. The first peak to elute (peak (i)) is fragment D which passes straight through. The remaining protein (peaks (ii) and (iii)) was eluted using a sodium chloride gradient of 0 to 0.5 M NaCl, to displace fragment E off the column. Peak (ii) contains a mixture of fragments D and E, whilst peak (iii) contains fragment E alone.

The next stage of these studies was the development of the two selected modes of investigation, ie. photosensitized radioactive surface labelling and photosensitized cross-linking. First of all, a set of conditions were established for the optimization of either method which led to consistent and reproducible results. Both methods have certain advantages in that they are carried out in solution, the initiation and duration of the reaction can be easily controlled, and the processes occur via highly reactive, short-lived, intermediates.

Initial photolabelling experiments showed that the binding of the radioactive tryptophan label to the protein could be induced by illumination in the presence of fluorescein. It was also shown that the linkage between the protein and label is covalent since bound label cannot be removed by, for example, dialysis, SDS-polyacrylamide gel electrophoresis, or denaturation by 8M urea. However, as seen previously (Chapter 3) photosensitized labelling studies also showed that a side-reaction which competes with the labelling occurs. Singlet oxygen generated when fluorescein is irradiated with near UV light reacts both with amino acid residues on the polypeptide chains of the protein as well as with the free [^{3}H]tryptophan in solution. The latter is converted to a free radical and reacts with the protein surface; covalent bonds are formed and the amount of radioactive tryptophan bound can be calculated. This information indicates which parts of the molecule are exposed to solvent and are therefore surface-orientated. However covalent

bonds between protein and free tryptophan are not the only ones that result from this reaction, protein-protein cross links are also generated. Thus in the case of labelling studies conditions have to be optimized such that the photolabelling reaction is the predominant one occuring. This involves such measures as decreasing the amount of protein used and decreasing irradiation time. The degree of cross-linking can easily be monitored by examining the protein on SDS-polyacrylamide gels.

However the potential of the cross-linking reaction itself was also realized and this technique was used independently as a means of studying the molecule's conformation. The technique of fluorescein-activated cross-linking follows similar chemistry and uses similar apparatus to the technique of photosensitized radioactive labelling with one major difference, - no free tryptophan is added to the system.. Consequently the singlet oxygen transfers its energy only to protein molecules which then form cross-links with any other protein (intermolecular cross-linking), or part of the same protein (intramolecular cross-linking). The cross-links are very short in length and thus cross-linked species must have been in very close proximity originally. Intramolecular cross-links occur within the molecule itself and provide information regarding the orientation of the chains. Intermolecular cross-links occur between protein molecules and provide information regarding surface orientation of the actual chains.

Once the main problems in the methodology had been tackled and overcome and the experimental conditions established, the next step was to investigate the photosensitized labelling and cross-linking of both fibrinogen and its plasmin derivatives, fragments D and E, under various conditions, with particular reference to the influence of calcium. However, prior to discussing the results, a comprehensive review of the different methods which have been used by various authors for the purpose of this investigation and the suggested models to date, (as outlined briefly in Chapter One), is necessary to bring this work into perspective.

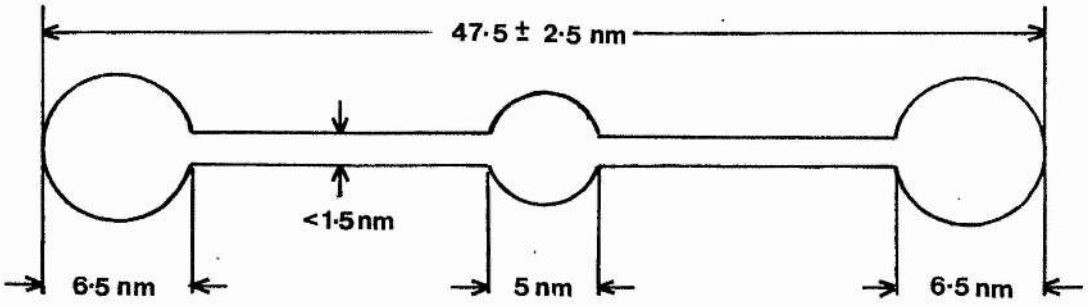
There has been considerable controversy over the years regarding the conformation of fibrinogen. Intact fibrinogen does not crystallize and therefore definitive X-ray crystallographic analysis has not yet been carried out. Crystals have been produced after limited cleavage of the molecule by a bacterial protease such as that from Pseudonomas aeruginosa (Tooney and Cohen, 1972), however this in itself suggests that the authors were examining a molecule which had undergone a significant conformational change. Consequently, most of the information regarding the shape of the molecule has been obtained by electron microscopy or physiochemical analysis.

Electron microscopic observations of fibrinogen have led to the proposal of several structural models ranging from an elongated trinodular structure, Hall and Slayter (1959), to a

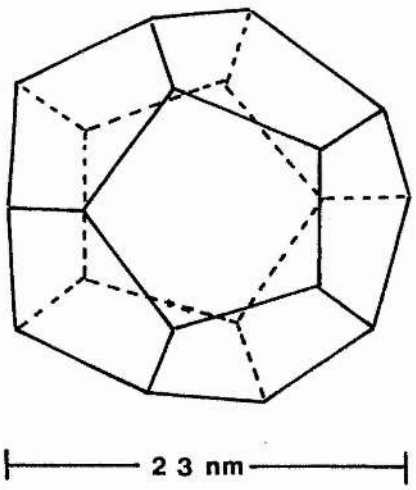
spheroidal shape, Koppel (1966) (see Figure 4-1). Hall and Slayter carried out their studies by imaging individual fibrinogen molecules that had been sprayed on mica in a volatile buffer, dried in vacuum, and shadowed with heavy metal. They proposed that the fibrinogen molecule consists of a linear array of three nodules held together by a very thin thread, the two end nodules being similar to each other but slightly larger that the central one. Most of the data from biochemistry and physical chemistry fits this model, however much controversy arose from laboratories attempting to visualise the molecules by negative staining which is generally recognised as a higher resolution technique than shadowing. Large globular particles were the predominant species found in these specimens (Koppel, 1966; Pouit et al, 1972;) and led Koppel to name the structure he observed a pentagonal dodecahedron. Pouit et al also suggested, however, that the globular model they put forward had the ability to unfold to a more rod-like structure under different physical conditions such as activation by thrombin or other agents.

Support for either of these two main models arose from a number of workers using various techniques and include refined versions of the two proposed conformations.

In 1979, Fowler and Erickson attempted to establish the correct model for the structure of the fibrinogen molecule by using their improved methods for both shadow casting and



Hall and Slayter (1959)



Koppel (1966)

Figure 4-1: Proposed models of fibrinogen conformation by Hall and Slayter (1959) and Koppel (1966).

negative staining in the same study. In the shadow casting technique, glycerol was included in the buffer in which the protein was sprayed onto the mica. It was found that in the absence of glycerol the protein was deposited in aggregrates or the molecular structure distorted by drying; in the presence of glycerol, however, most of the droplets had clean areas of unaggregated molecules. With respect to the negative staining technique, the authors found that the standard carbon films used were very hydrophobic and the stain would not spread unless the fibrinogen solution was applied at high protein concentrations, leading to the random aggregration of molecules. In order to get the negative stain to spread and stick to the carbon film at low protein concentrations, the authors used a surface of the film that had been rendered hydrophilic by the "flotation technique" (see reference) and were thus able to prepare specimens at concentrations of 1-5μg/ml, 1000 times less than those used in the previous studies of Koppel (1966) and Pouit et al (1972). The end result showed that molecules examined by either method had a trinodular structure of dimensions very similar to those of Hall and Slayter, (ie. length = 45nm ± 2.5nm, outer nodule diameter = 6-7nm, central nodule diameter = 4-5nm). The authors also suggest that globules seen in various negative staining studies are aggregates of these molecules. This point had also been suggested by Krakow et al (1972). The work of Williams (1981,1983), who used electron microscopy after contrast enhancement by rotary shadowing and negative staining to examine

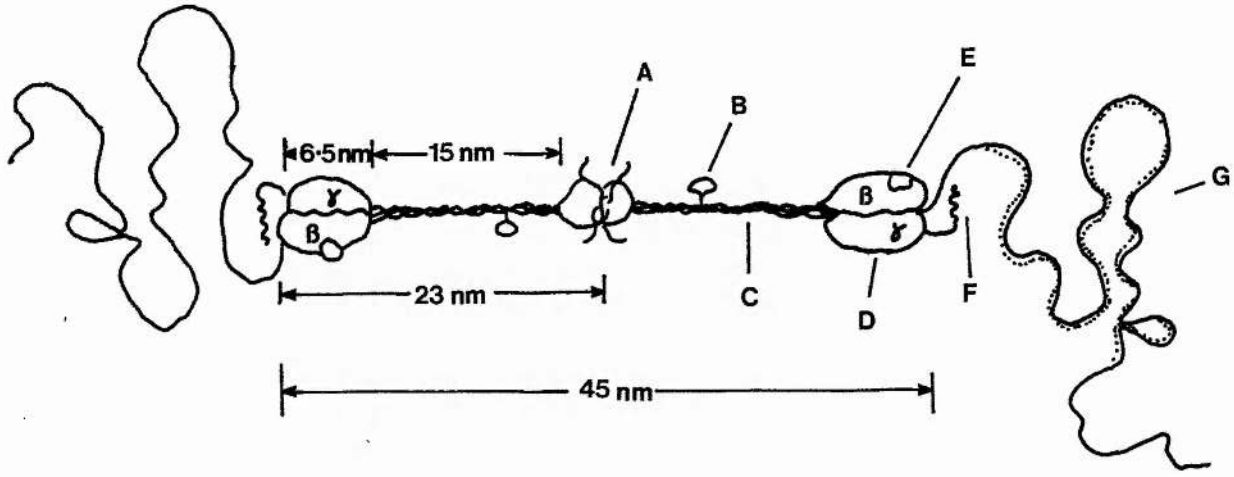
the conformation of fibrinogen, also showed the molecule to have a trinodular structure.

Even though digestion of fibrinogen by the action of Pseudonomas proteases on the Aα-chain results in crystals of the partially degraded molecule, the overall structure seen by electron microscopy images and X-ray crystallography is basically trinodular (Weisel et al, 1981, 1985; Cohen et al, 1983).

One of the strongest arguments in favour of the trinodular model comes from the asymmetric cleavage model by plasmin digestion (Marder et al, 1969). The authors purifed the various digest fragments and their physiochemical and immunological properties were defined: they found that the core fragments D and E correspond to the outer and central nodules respectively, fragment Y is binodular comprising one D and one E, and fragment X is a trinodular molecule missing the carboxyl-terminal segment of the Aα-chain. These findings were also confirmed by electron microscopic analysis of the fragments (Fowler et al, 1980; Adams-Lucas et al, 1983;) where the relationship of each plasmin cleavage fragment to the trinodular model of Hall and Slayter was defined. A number of other laboratories have used electron microscopy of antibody-labelled fibrinogen as a method of investigation. Telford et al (1980) used antibodies to the disulphide knot fragment (N-DSK) of fibrinogen in combination with metal shadowing to locate its

site within the intact molecule and concluded that this lies within the central nodule of the trinodular model. Price et al (1981) localized the D and E domains in the outer and middle regions of the trinodular fibrinogen molecule respectively, using specific anti-D and anti-E Fab fragments in metal shadowed specimens. Similar results were also reported by Norton and Slayter (1981).

The model of Doolittle et al (1977) also supports the trinodular conformation. This was generated by computer simulation based on both primary structure data and by taking into account the evolutionary perspective (see Figure 4-2). Basically the model consists of two terminal domains tethered to a central domain by two three-stranded ropes thought to be in the form of coiled coils in which the non-polar residues are turned inwards and the polar side chains extend out into the aqueous solvent (Doolittle et al, 1978); the C-terminal parts of the Aα-chains are freely arranged in space. The model was originally (1977) drawn with a bend because of the presumed parallel nature of the disulphide bonding between the two dimeric halves, similar to the situation found in immunoglobulins. However in the light of the work of Hoeprich and Doolittle (1983), who determined that the two γ-chains, and hence the dimeric halves, were connected in an anti-parallel manner, this constraint was lifted. This model thus leaves the question of the molecule's flexibility open to speculation.



A = central domain including fibrinopeptides
B = γ chain carbohydrate cluster
C = "coiled-coils" interdomainal connector
D = terminal domain consisting of homologous β and γ chain segments
E = β chain carbohydrate cluster
F = γ chain cross-linking site
G = α chain carboxy terminal extension

Figure 4-2: Updated schematic depiction of the vertebrate fibrinogen molecule postulated by Doolittle et al (1977). From: Doolittle (1983).

The question of the flexibility of fibrinogen molecules is still an issue. It is not hard to envisage that one or more of the components of this multidomainal structure can bend or twist independently to other parts of the molecule. Various experimental data, including electron microscopy and immunochemical studies, has been put forward which supports the concept of a flexible fibrinogen conformation.

Bachmann et al (1975), in a study of freeze-etched fibrinogen, proposed a cylindrical, rod-like molecule with rounded ends, 45nm ± 1.5nm long and with a diameter of 9nm ± 1nm. These dimensions are roughly compatible with the trinodular model, though this technique apparently lacks sufficient resolution to identify the individual domains. Approximately 30% of the observed molecules were bent to various degrees, giving the structure a certain degree of flexibility. Estis and Haschemayer (1980) also reported that fibrinogen (negatively stained with sodium phosphotungstate) had the form of a flexible, indistinctly beaded rod, having a nodular configuration when observed unstained.

Mosesson et al (1981) investigated the conformation of fibrinogen by evaluating scanning electron microscopic images of the molecule. Their findings were consistent with the trinodular structural model (length = 46nm ± 2nm), however it was also suggested that the molecules are flexible and may exist in unfolded configurations or as relatively compact, partially or

completely folded forms. Further studies by this group (Wall et al, 1983) yielded similar results.

The proposed model of Hudry-Clergeon et al (1975), based on the results of co-workers Pouit et al (1972), is that of a non-compact sphere, 22nm in diameter, very similar to that of Koppel. These workers proposed that the constituent chains of the molecule fold around the N-terminal disulphide knot and are stablized by intramolecular reactions of an electrostatic nature. However it was also suggested that the latter interactions could be modified by enzymatic peptide cleavage or by the action of certain paracoagulant factors, thus allowing the uncovering of sites involved in fibrin polymerization and cross-linking and giving the molecule a certain amount of flexibility. It was suggested that the globular shape only persists in the first aggregation stages of fibrin formation and the structure of the fibrin monomer included in the fibrin fibre is less connected with the one of the native fibrinogen molecule. The workers maintained, however, that the spherical shape would be predominant in physiological conditions (see Figure 4-3).

Marguerie et al (1975) and Marguerie and Stuhrmann (1976) were the first to investigate the structure of fibrinogen by neutron small angle scattering. The resulting structure from the analysis pattern in terms of multipoles was consistent with that of an ellipsoid or flattened disc, more graphically termed as

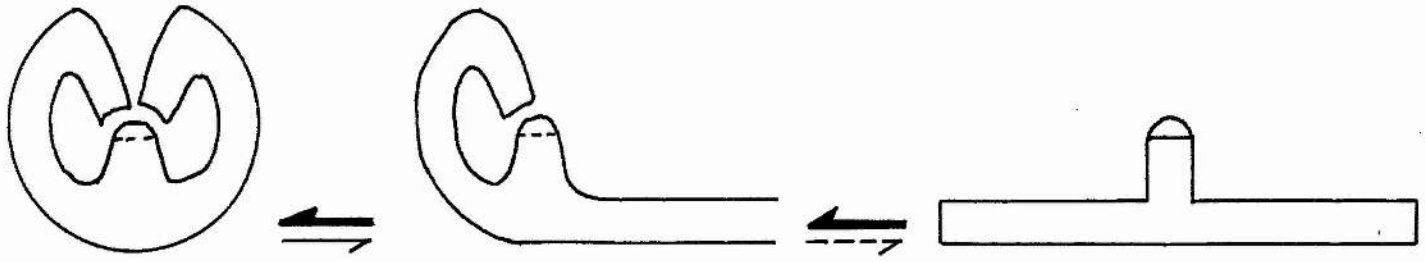


Figure 4-3: Different possible forms of the fibrinogen molecule proposed by Hudry-Clergeon et al (1975).

the 'banana-shaped' model. The authors proposed the possible localization of three structurally important zones as well as flexible zones within the molecule. The model agrees with the concept of a highly hydrated protein, - 6g H_2O/g protein was the estimated degree of hydration. On the basis of this work the authors also suggested that the bent structures observed by Bachmann et al (1975) represented the native form of fibrinogen rather than denatured molecules and that Koppel (1966), using the negative staining method, had observed loosely packed flattened discs rather than spheres (Marguerie, 1979).

From his small angle X-ray scattering data Lederer (1979) suggested that, in fact, fibrinogen is a highly flexible 'sausage-like' molecule (45nm long, 9nm wide) not having just one specific conformation. The author also proposed that the disc-like model of Marguerie and Stuhrmann (1976) merely represents one specific conformation of this highly flexible molecule. Serralach et al (1979) attempted to reconcile experimental and calculated hydrodynamic data with electron microscopic models of fibrinogen. These workers found poor agreement between the two, even if the degree of hydration was varied upto 9g H_2O/g protein. However agreement was achieved by assuming a highly flexible molecule. The idea of flexibility was further postulated by Hantgan (1981,1982) in his conformational studies of fibrinogen in solution using steady-state fluoresence polarization. His results indicate that fibrinogen is not a rigid molecule, but can undergo bending or twisting motions in

solution, and strongly support some kind of segmental flexibility within the molecule.

Plow and Edgington (1982) used specific antibody probes to investigate the surface topography of fibrinogen in its native state. The described epitopes were superimposed on the trinodular model proposed by Doolittle et al (1977) and it was found that these were better accommodated by a more flexible model, or 'flexion', in which the D and E domains could associate. The authors suggested an equilibrium between flexed and linear formats, with only the latter conformation capable of fibrin polymerization.

As well as the flexibility of the molecule, another issue regarding the conformation of fibrinogen is the spatial arrangement and role of the C-terminal parts of the Aα-chains.

Doolittle et al (1979) divided the amino acid sequence of the Aα-chain into three zones, namely ZN, ZM and ZC. These correspond to the amino-terminal third (residues 1 - 239), the middle third (residues 240 - 424), and the carboxyl-terminal third (residues 425 - 610) of the Aα-chain respectively. A short subzone (constituting part of the ZN region, ie. residues 195 - 239) was designated IZ. Residues 1 - 82 and 105 - 200 of the ZN region correspond to segments of the Aα-chain found in the E and D domains respectively (Henschen et al, 1982).

In the model of fibrinogen postulated by Doolittle et al (1977) the C-terminal parts of the Aα-chains are freely arranged in space (see Figure 4-2). This suggestion is supported by Price et al (1981) who, however, used Fab fragments of antibodies specific only for the ZM portion of the Aα-chain in their investigation. From this work these authors intrepreted their results as showing these portions of the Aα-chain and (by inference) also the C-terminal parts, to be situated beyond the outer domains of the molecule, at a considerable distance from the central domain. From observations derived using a novel electron microscope specimen preparation technique, Rudee and Price (1981) also suggested that the Aα C-terminal extensions protrude away from the D domains of the molecule. On the other hand, Telford et al (1981), using antibodies to fragment H of fibrinogen (which roughly corresponds to the ZM portion of the Aα-chain), found that this is located on the surface of the intact molecule, in association with the D domain.

However in 1981 the investigations of Mosesson et al led to the suggestion of another model which, although similar to that of Doolittle, differs in the spatial arrangement of the C-terminal parts of the Aα-chains. The authors suggested that the latter regions of these chains are situated within the mass integration radius for the central domain. Thus, in this model, the C-termini of the Aα-chains protrude from the outer domains and extend towards the central domain by winding around the long axis (see Figure 4-4). This view is supported by the fact that

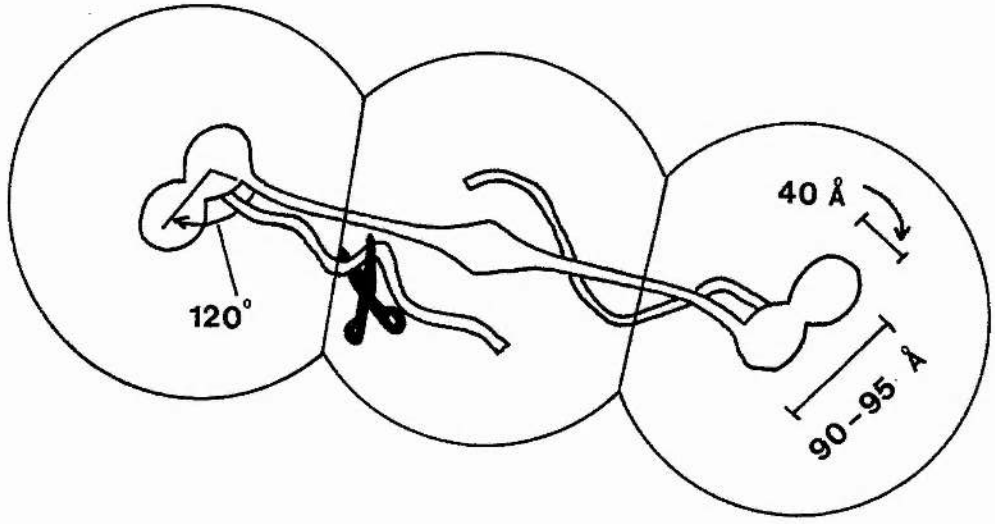


Figure 4-4: Schematic model of the fibrinogen molecule postulated by Mosesson et al (1981).

The scissors indicate a site on the Aα chain, cleavage of which results in molecules characteristic of fraction I-9.

this structural feature is observed in negatively stained I-4 fibrinogen preparations but not in I-9 where the C-terminal parts of the Aα-chains are removed.

From their calorimetric study of the melting of fibrinogen and its fragments, Privalov and Medved' (1982) showed that parts of the native molecule which were removed in the early stage of its proteolytic degradation have a cooperative structure which melts on heating, with a noticeable heat effect. This effect was not seen in the melting profile of the X fragment when compared to that of intact fibrinogen. Further calorimetric work carried out by Medved' et al (1983) supports the model put forward by Mosesson and goes further to state that the C-terminal parts of the Aα-chains form two structural domains which strongly interact with each other in the region of the central domain of the molecule, probably by means of some complimentary surface sites. Computer analysis of the amino acid sequence of the C-terminal two-thirds of the Aα-chain (ie. residues 200 - 610) showed that α-helix and β conformation are quite probable in the 390 - 550 residue section of the considered polypeptide, while the 250 - 390 residue section is likely to be in random conformation. The association of these two structural domains results in what can be termed as the 'fourth domain' of fibrinogen.

Erickson and Fowler (1983), using electron microscopy, also observed the existence of a fourth nodule (5nm in diameter) in

the fibrinogen molecule, which is located near the central domain, 10nm away on one side of the molecular axis. Since the nodule was never observed for the X fragment, the authors suggested that it is formed by the C-terminal portions of the two Aα-chains.

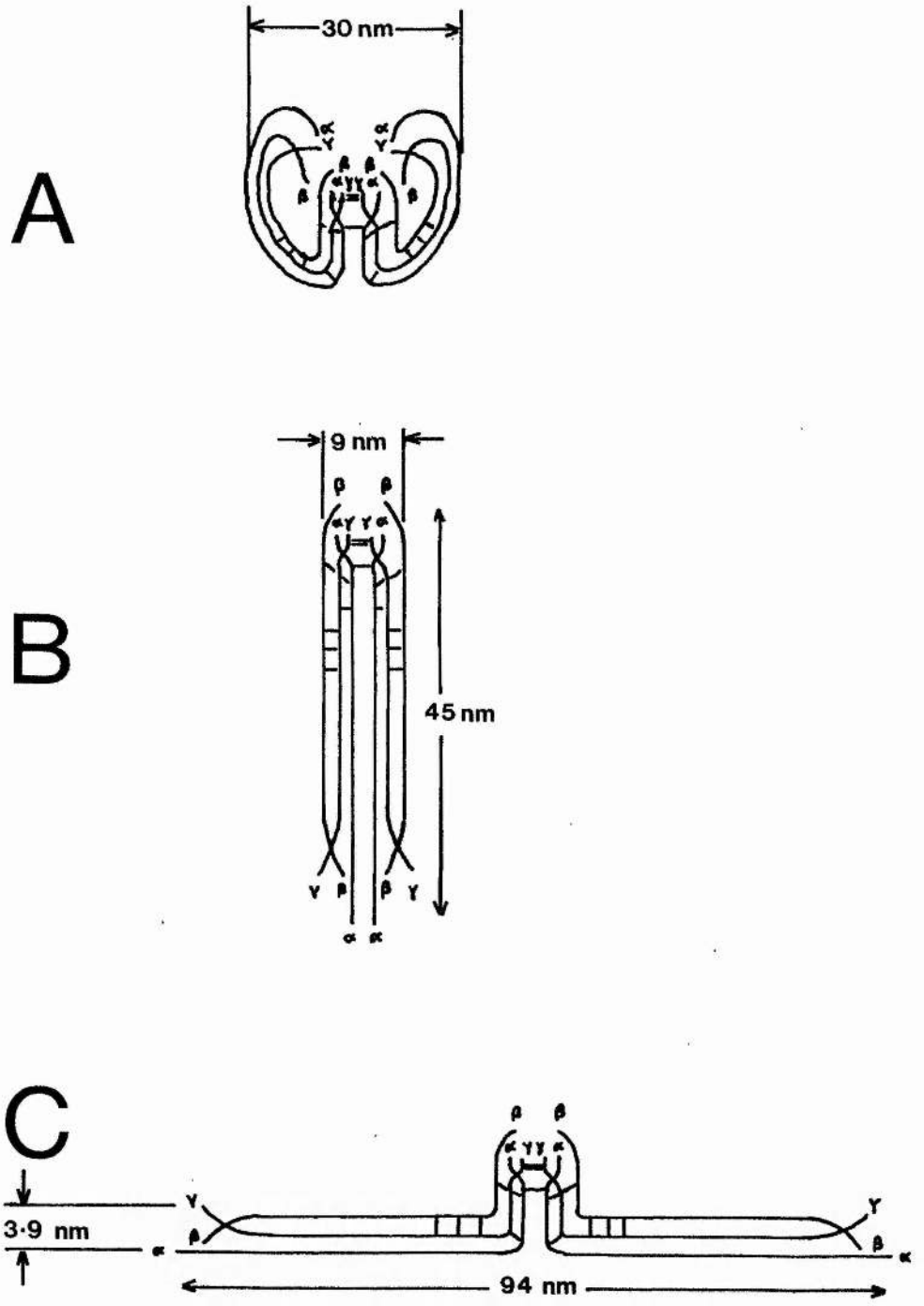
Cierniewski et al (1984) investigated the conformation of the C-terminal regions of the Aα-chains by immunochemical analysis. The authors found that epitopes expressed by the intact Aα-chain, which were occult in the native molecule, became accessible when fibrinogen was cleaved with plasmin. The authors thus proposed that their results are consistent with structural models depicting the C-terminal portions of the Aα-chain as being systematically ordered in the native fibrinogen molecule. Significantly, the authors suggested that the existence of such ordering introduces a new variable into regulation of the many functions of this region of the molecule.

Weisel et al (1985) also demonstrated the existence of the fourth domain. These authors showed that molecules modified for crystallization by limited cleavage with a bacterial protease retained the main features of the native structure. However the major difference between the modified and native fibrinogen was the loss of a small central domain which they suggest is formed by the C-terminal portions of the Aα-chains folding back to interact at the centre of the molecule.

There are a number of possible reasons for the plethora of models put forward, one being that investigation of the structure was done using a wide variety of techniques. Electron microscopy generally involves rigid preparative procedures which may affect the native conformation of fibrinogen. Artefacts easily occur using this method and differences in the procedure used greatly influence the state in which fibrinogen is observed. On the other hand, workers using physiochemical analysis as a tool for investigation may have obtained over-simplified results since the effects of different regions of the molecule are usually averaged out in the interpretation, thus leading to ambiguity. Different methods of fibrinogen purification were also implemented by the various workers, thus resulting in fibrinogen species of different intactness and quality for examination.

Bang (1964) and Krakow et al (1972) also showed that the fibrinogen molecule exhibited an abnormally high degree of hydration and this could explain some of the differences in results obtained by the various methods. Different degrees of hydration could have resulted in different interpretations to fit either of the two diametrically opposed models. Hydrodynamic and solution data can fit either model: if the molecule is a narrow or trinodular structure 45nm long it will be a relatively compact protein, whereas if the molecule is a sphere 20nm in diameter, it must be very highly hydrated (approximately 8g H_2O per g protein).

However another very important source of differences is the influence of the particular enviroment on the molecule's conformation. Each investigation was usually carried out at fixed conditions of, for example, buffer and pH, and thus the resulting structure holds true only for the parameters under which the experiment was performed. Hudry-Clergeon et al (1975) proposed that the fibrinogen molecule could exist under different balanced forms, the relative properties of which depend on various factors such as temperature, pH, ionic strength, solvent nature and protein concentration. Another group of workers, Mueller and Burchard (1978), investigated the structure of fibrinogen using light scattering. Although they found that under the fixed conditions in which their experiments were carried out the molecule appeared to have a rod-like conformation, the authors postulated that fibrinogen can exist in different interchangeable isomeric forms such as a folded rod similar to that of Bachmann et al (1975) and the banana-shaped model of Marguerie et al (1975) (see Figure 4-5). More importantly, however, these authors suggested that the predominant isomer would be dependent on its enviroment, with special emphasis on the influence of calcium. The importance of calcium on the structure of fibrinogen has also been emphasised by Kemp (1984). The author suggested that since fibrinogen is able to bind calcium ions with both high and low affinity and that these ions are important to the integrity of the molecule, work done in the absence of calcium may not yield a valid interpretation of the structure.



A = structure similar to the model of Marguerie et al, (1975).
B = structure similar to the model of Bachmann et al, (1975).
C = rod-like conformation observed by Mueller and Burchard (1978).

Figure 4-5: Different isomeric structures of fibrinogen suggested by Mueller and Burchard (1978).

The importance of calcium on the structure and function of fibrinogen has been investigated by a number of authors whose work will be discussed in the course of this chapter. An example of one such study is that carried out by Larsson et al (1987), who studied fibrinogen by means of dynamic laser light scattering. Their results suggest that fibrinogen expresses a certain degree of flexibility in the absence of added calcium (but where calcium high affinity sites have not been vacated), whereas it is straightened or unfolded when calcium is added to the system. More specifically the authors proposed that when no calcium is present fibrinogen resembles a folded rod, similar to the structure of a disc and not unlike the proposed oblate ellipsoid model of Marguerie and Stuhrmann (1976); however in the presence of calcium the molecule unfolds to a straight rod structure or prolate ellipsoid similar to that observed by Bachmann et al (1975). Larsson et al also suggested that as calcium is known to enhance the rate of fibrinogen to fibrin formation, this stabilization of the fibrinogen molecule by calcium may be relevant for enhancement of the polymerization rate. This may by brought about, for example, by exposure of polymerization sites in the molecule.

In the studies presented in this work, fibrinogen and its derivative core fragments were examined under different solvent conditions so as to test the premise put forward by various authors that the molecule is a flexible one, existing in different isomeric forms. The importance of tightly bound

calcium ions to fibrinogen has been investigated (Marguerie et al, 1977), but the role of the larger number of low affinity sites is still not clear. Consequently the conformation of the molecule was examined with particular reference to the influence of calcium.

Preliminary experiments suggested that fibrinogen is a molecule whose conformation is influenced by its enviroment. This effect was seen when the protein was found to be more open and thus more prone to interaction with other molecules in the Tris/$CaCl_2$ buffer compared to the Tris and NaCl/phosphate buffers (all at pH 7.5). This effect was observed as a result of using both photolabelling and cross-linking techniques. The presence of calcium was found to be a major cause in the unfolding of the molecule.

When the distribution of label into the three constituent chains of fibrinogen (Aα : Bβ : γ) was calculated for the above buffers, results showed that in both NaCl/phosphate and Tris buffers the Aα and γ-chains are equally exposed. However in the Tris/$CaCl_2$ buffer the Aα-chain is the most surface-orientated and takes up almost as much label as the Bβ and γ-chains combined. This enhanced surface-orientation of the Aα-chains in the presence of calcium suggests that this conformational change may be linked to this particular part of the molecule having some functional role under the conditions described.

Results derived from cross-linked fibrinogen in the three different buffers correlate with those obtained from labelling experiments. Here calcium was seen to increase the cross-linking potential of the molecule since intermolecular cross-linking is more extensive in the Tris/$CaCl_2$ buffer compared to both Tris and NaCl/phosphate buffers. This opening up of the molecule brought about by calcium is similar to the effect first suggested by Pouit et al (1972), ie. that the molecule 'unfolds' under certain solvent conditions. Larsson et al (1987) showed that calcium plays a key role in this conformational change; this view is consistent with the results presented in this work. In addition, analysis of gels (5% acrylamide) of reduced fibrinogen after cross-linking showed that the Aα-chain is the subunit which is taken up by cross-linking the most rapidly. This correlates with photoaffinity labelling results where the Aα-chain was seen to be the most surface-orientated of the chains in the presence of calcium, and would thus be the most likely subunit to be taken up in cross-links first. Full digestion of the cross-linked samples showed that the D and E core domains are not involved in the conformational changes seen to occur in the different buffers.

It is widely known that calcium is important to the structural integrity and function of fibrinogen. As seen earlier, calcium ions bind to fibrinogen (Marguerie et al, 1977), stabilize it against denaturation by heat (Ly and Godal,

1973) and acid (Marguerie, 1977), and limit its digestion by proteases such as plasmin (Haverkate and Timan, 1977).

Various authors have also shown that calcium ions accelerate the conversion of fibrinogen to fibrin by increasing the rate of fibrin polymerization. (Boyer et al, 1972; Endres and Scheraga, 1972; Brass et al, 1978;). The importance of calcium in fibrin formation has also been studied by Hardy et al (1983). These authors suggested that high-affinity bound calcium forms an integral part of the fibrinogen structure while free calcium ions have a regulatory role in fibrinogen conversion to fibrin by modulating the rate of polymerization and not that of fibrinopeptide cleavage.

Okada and Blomback (1983) demonstrated that calcium ions have a pronounced effect on fibrin gel structure. The authors showed that the rigidity of the gel is increased when it is formed in the presence of calcium. In addition, the authors suggested that this structural change is explained by the binding of calcium to low affinity sites on fibrinogen or the fibrin monomer/polymer. The authors also found that although magnesium interacts with fibrinogen, its effect is different from that exerted by calcium. Although magnesium decreases the clotting time of the gel-forming system, no effect on the k_s (permeability coefficient) values of the fibrin gels formed in the presence of this ion was seen.

Ruf et al (1986) showed that calcium ions do not influence the release of fibrinopeptides A and B in the initial enzymatic phase of the reaction. However during subsequent polymerization, desA-fibrin polymers formed in the presence of calcium ions represent better substrates than those formed in the absence of calcium for the proteolytic attack of thrombin on the Bβ-chain. The authors suggested that this can be explained either by an accelerated formation of fibrin oligomers in the presence of calcium, or by formation of different oligomers due to calcium ions serving as a better substrate for the attack of thrombin on the Bβ-chain.

In addition, as seen earlier on in this chapter, calcium ions are essential for the activity of factor XIII in fibrin cross-linking. (Lorand and Konishi, 1964; Curtis et al, 1974;).

From the information already gathered as a result of this study it can be seen that fibrinogen is a dynamic molecule whose flexibility is influenced by the presence of calcium. The two techniques of photosensitized radioactive labelling and photosensitized cross-linking have been shown to have definite applications in their use as probes into the conformation of the molecule. Results derived from the above methods of investigation are not only reproducible but also support one another. The next set of experiments was done to investigate the effect of calcium on the molecule in greater detail. The range of calcium concentrations selected encompassed both the

physiological level (ie. approximately 10^{-3}M) and the dissociation constant of the low affinity binding sites ($K_d \approx 10^{-3}$M).

Results derived from gels of cross-linked fibrinogen showed that the amount of intermolecular cross-linking increased with an increase in the calcium concentration. This implies that fibrinogen is more compact at lower calcium concentrations and therefore less prone to contact with other molecules. Analysis of gels of reduced, cross-linked, fibrinogen showed that the increase in the proportion of intermolecular cross-linking occurs mainly through the Aα-chains. Thus one can visualize the unfolding of the molecule due to the influence of calcium, with the C-terminal portions of the Aα-chains playing a leading role in the formation of intermolecular links.

This conclusion is supported by results from further work done in our laboratory where fibrinogen was progressively digested to fragment X and then cross-linked at intermediate stages (Webster, 1987). In this experiment the amount of intermolecular cross-linking decreased as the digestion progressed and did not occur to any significant extent with fragment X.

In this study, the samples cross-linked at the different calcium concentrations were then fully digested in order to investigate whether the core domains are involved in the

observed conformational changes. From the resulting gel patterns it was concluded that the D and E domains do not interact as a result of the influence of calcium.

The flexibility of fibrinogen and the effect calcium has on the molecule's conformation was also seen in the photolabelling experiments. As the concentration of calcium is increased in the millimolar range, the amount of label incorporated into the molecule increases, suggesting that fibrinogen adopts a more open conformation in the presence of calcium. The change in conformation occurs over a range encompassing both the physiological calcium concentration and the dissociation constant of the low affinity binding sites. The saturation of these sites is therefore important and, as has been suggested by various authors, may have a regulatory function. The effect is biphasic with an increase in surface area accompanying an increase in calcium concentration up to 2mM (phase I) and a further unfolding of the molecule occuring above approximately 3.5mM $CaCl_2$ (phase II). The dip in incorporation which occurs between the two phases (from here on referred to as the 'intermediate' phase) has its own significance and will be discussed later.

Digestion of photolabelled fibrinogen to the core domains, D and E, showed that the labelling of the terminal and central domains is affected but not identically. Incorporation into the D domains is influenced in both phases and dips in the

intermediate phase, whereas that of the E domain is mainly affected in phase II. As expected, the sum of the labelling of the core domains is also directly reflected in that of the whole molecule.

The results show that the E domain does not constitute a major part of the molecule's surface at the lower end of the calcium concentration range examined. However as the calcium concentration is increased, incorporation into the E domain also increases. This could be due either to a conformational change within the domain or to its uncovering by movement of some other part of the molecule.

Two regions of the molecule that may possibly occlude the E domain are (a) the D domains or (b) the C-terminal portions of the Aα-chains. Digest patterns of cross-linked fibrinogen indicated that the D domains are not directly involved in calcium-induced conformational changes of the molecule. However if the percentage of Aα-chain that is not taken up by cross-linking is compared to the degree of labelling incorporated into the E domain, it can be seen that the two are interrelated. Greater surface labelling of the E domain was reflected by the disappearance of uncross-linked Aα-chains, both effects increasing as the calcium concentration was raised. This suggests that increased incorporation into the E domain is due to the uncovering of this region by the C-terminal parts of the

Aα-chains, rather than the effect of a conformational change occuring in the domain.

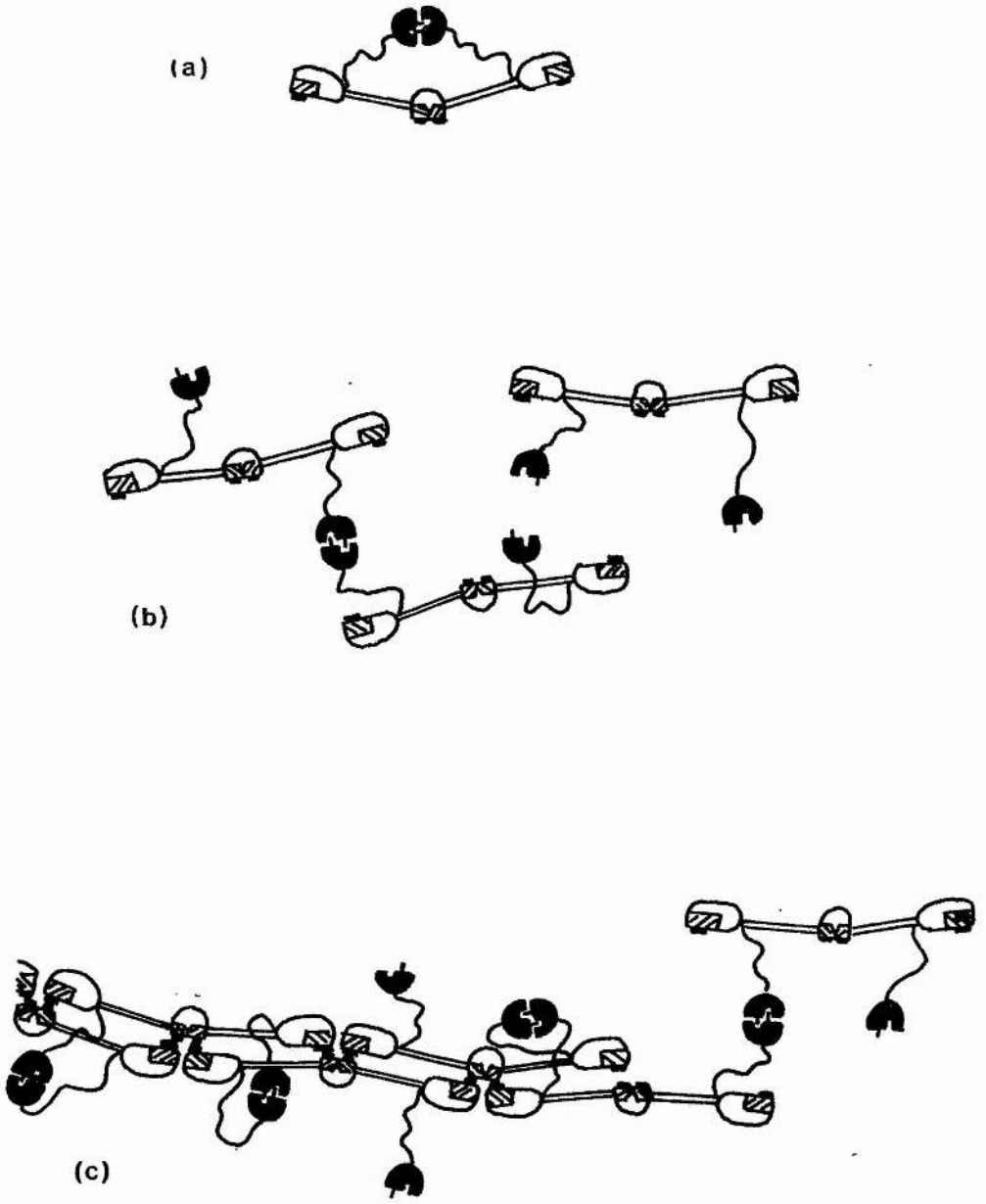
Thus from the results presented it is suggested that at low calcium concentrations the C-terminal parts of the Aα-chains are in close association with the central domain. This correlates well with the work of Mosesson et al (1981), Medved' et al (1983), and Erickson and Fowler (1983) regarding the spatial arrangement of the C-terminal portions of the Aα-chains in the native fibrinogen molecule. It should also be noted, however, that none of the above authors used buffers containing added calcium in their investigations. Consequently it can be assumed that the low affinity calcium binding sites of fibrinogen were not saturated in the above studies.

The ordered arrangement of the C-terminal parts of the Aα-chains raises the question of the functional role of this dimeric block. It is widely known that fibrinogen interacts with other plasma proteins and particles. The C-terminal regions of the Aα-chains contain several sites of interaction: other molecules involved include fibronectin (Mosher, 1975) and factor XIII (Credo et al, 1981), as well as platelets (Hawiger et al, 1982). Proteolytic damage to C-terminal portions of the Aα-chains also leads to the disruption of normal fibrin polymerization (Phillips, 1981). Clot opacity studies by the latter author suggest that this region of the molecule plays an

important role in lateral associations during fibrin polymerization.

Holm et al (1985b) also found that the C-terminal of the α-chain is important for fibrin polymerization. Their investigation of the plasma fibrinogen fractions HMW (molecular weight: 340,000, containing intact Aα-chains) and LMW (molecular weight: 305,000, containing just one intact Aα-chain) showed that HMW fibrin momomers polymerize at a higher rate than LMW monomers. The prolonged clotting time of LMW was found to be due to retarded polymerization and not to the enzymatic phase of coagulation (measured as release of fibrinopeptide A during incubation with thrombin). The authors also found that both types of monomer polymerize more rapidly in the presence of calcium.

Medved' et al (1985, 1986), however, proposed an additional role for the dimeric block they had observed in their earlier studies (1983), ie. that the Aα domains participate in fibrin polymerization not as structural components, but as a factor promoting the ordered process of fibrin assembly (see Figure 4-6). On the basis of their experimental evidence from electron microscopy and light scattering, the authors suggested that interactions between αC-domains can occur intermolecularly as well as intramolecularly and that the former predominate in monomeric fibrin compared to fibrinogen. The C-terminal parts of the Aα-chains are thus thought to dissociate from each other



(a) = fibrinogen
(b) = monomeric fibrin
(c) = the protofibril

αC-domains are denoted by black; the main active sites of the central (E) and peripheral (D) domains are shaded.

Figure 4-6 : The role of the αC-domains in fibrin assembly. (Medved' et al, 1986).

during fibrin formation, thereby acting as 'antennae' to help promote the rapid finding and oriented mutual approach of other fibrin monomers. This permits the main polymerization sites present in the central and peripheral domains to interact rapidly and accurately in the construction of protofibrils. In addition, the authors also suggested that the αC-domains accelerate lateral aggregation of the protofibrils in the second stage of fibrin polymerization. The results presented in this thesis support this hypothesis and further propose that calcium ions are the cause of the dissociation of the intramolecular Aα domain. This is so since, as demonstrated in the results, as the calcium concentration is increased, the dissociation of the fourth domain is triggered, thus enabling the E domain to incorporate more radioactive label.

Apart from its role in fibrin polymerization, this proposed activation of the molecule by calcium has several implications when the other functions of the Aα C-terminal regions are considered. It may well be that the Aα fourth domain occludes crucial sites of interaction and that calcium may be the factor required to 'activate' these sites by its effect on the dissociation of the Aα domain.

One of the molecules involved in the interaction of the C-terminal regions of the Aα-chains is factor XIII'. Credo et al (1978) showed that fibrinogen enhances the generation of fibrinoligase activity by lowering the calcium requirement of

the thrombin-modified factor XIII zymogen (ie. factor XIII', or a'_2b_2). The addition of a physiologically equivalent concentration of fibrinogen promoted the conversion of factor XIII' to the functionally competent enzyme factor XIIIa (ie. a_2*) at physiological calcium concentrations. In the absence of fibrinogen approximately 6 - 10mM Ca^{++} would be needed to achieve the same effect. The authors later suggested (1981) that fibrinogen thus functions as a physiologically important calcium-modulator protein. These workers showed that the critical interaction domain for contact with factor XIII' corresponds to a stretch of residues in the middle section (ie. residues 243 - 476) of the Aα-chain of fibrinogen. Proteolytic damage to C-terminal portions of the Aα-chains leads to a loss in the ability of fibrinogen to accelerate the activation of factor XIII'.

Fibronectin (otherwise known as 'cold-insoluble globulin') is a large glycoprotein (molecular weight: 440,000) comprising a dimeric molecule with carboxyl termini linked in the central region by a disulphide bond, and an amino terminus at each end of the strand. Purified fibronectin is always found to contain a small amount of fibrinogen as a contaminant or co-purifying species. Mosher (1975) showed that factor XIIIa cross-links fibronectin to fibrin or fibrinogen and demonstrated that attachment was to the α-chain of fibrin. (The cross-link acceptor site on fibronectin was identified as a glutamine, three residues from the amino terminus of the chain). In

addition it was found that fibrinogen loses its ability to bind to fibronectin if the C-terminal parts of the Aα-chains are damaged (Stahakis et al, 1978). Niewiarowska and Cierniewski (1982) also reported that the incorporation of fibronectin into fibrin is not only dependent on fibronectin concentration and temperature, but also on the presence of millimolar calcium.

The interaction of fibrinogen with specific receptors on the surface of the activated platelet results directly in platelet aggregation. Binding occurs only when platelets are stimulated with certain agonists such as ADP, epinephrine and thrombin. The native structure of fibrinogen is also a prerequisite for optimal binding and aggregation and it has been shown that fragment X digests are less potent in supporting aggregation compared to fibrinogen (Niewiarowski et al, 1977). The presence of calcium, necessary for receptor regulation and receptor-ligand interaction, favours the specific conformation for optimal function.

Three different fibrinogen domains have been implicated in the fibrinogen-platelet interaction, namely the amino terminal regions of the Aα and Bβ-chains, the carboxyl terminal regions of the γ-chains, and the carboxyl terminal regions of the Aα-chains. The involvment of the latter domain is of specific relevance to the studies presented in this work.

Hawiger et al (1982) reported that aggregates of the Aα-chain support ADP-induced platelet aggregation. In addition, Marguerie et al (1984) found that photoactivation of fibrinogen (which had been modified with the heterobifunctional reagent, SANAH, ie. N-succinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate)) caused the protein to become covalently bound to the platelet through the carboxyl terminal regions of the Aα-chains. Fibrinogen missing the extreme carboxyl termini of the Aα-chains failed to cross-link to platelets efficiently under similar conditions, indicating that the integrity of this region of the chain is essential for cross-linking to occur. The authors also showed that the Aα-chain comes into close proximity to the platelet membrane glycoprotein IIb/IIIa complex on binding to the platelet. The implication of the involvement of the latter heterodimer complex in fibrinogen receptor function was also demonstrated by Bennett et al (1982).

Divalent ions are necessary for binding to occur. Fujimura and Phillips (1983) found that the membrane glycoproteins IIb and IIIa exist as a complex in vivo and that this is regulated by the presence of calcium ions. In addition, Phillips and Baughan (1983) found that fibrinogen binding to platelets was optimum at 10^{-3}M calcium. It is interesting to speculate that since, as seen in these studies, calcium has been shown to trigger the dissociation of the fourth domain, the presence of this divalent ion may thus be necessary to uncover sites of

interaction on the Aα-chains which are involved in platelet interactions.

The influence of calcium was also seen to affect the incorporation of label in other parts of the fibrinogen molecule. Incorporation into the D domain is affected in both phase I and II, with a decrease in incorporation occuring in the intermediate phase. This incorporation profile could be the result of either conformational changes occuring within the domain or to uncovering /covering of the domain by some other part of the molecule. In order to distinguish between the two, the effect of calcium on the surface labelling of fragment D was compared to results for the D domain.

Results showed that in phase I the available surface of the D domain increases, but that it decreases in the intermediate phase. The available surface of this region of the molecule then remains steady in the first part of the second phase but slowly increases again as the calcium concentration is raised further. On the other hand, incorporation into fragment D is relatively constant upto approximately 3.5mM $CaCl_2$, indicating that any changes in the incorporation of the domain upto this calcium concentration are not conformational but are due to uncovering (phase I) and covering (intermediate phase) of this region by some other part of the molecule (or molecules). However although a sharp increase in the incorporation of the fragment occurs in the second phase, (at a concentration of approximately 3.5mM to

5mM $CaCl_2$), incorporation in the D domain is relatively constant indicating that this region is still occluded at this stage. Thus the conformational change that occurs in the fragment, which renders this region of the molecule more open and thus more accessible to radioactive label, is masked in the D domain due to this region's occlusion by some other part of the molecule.

This observed calcium-induced conformational change is in accordance with the findings of several authors. As seen previously, Mosesson et al (1981) investigated the structure of fibrinogen by scanning transmission electron microscopy and results from this study were consistent with tridomainal structural models. However the authors found that the outer terminal domains of negatively stained molecules could be resolved into at least two discrete subdomains forming an oblong structure, usually at an angle of 120° - 150° relative to the long axis (see Figure 4-4). Williams (1981) also observed these oblong terminal protrusions (length = 9nm; width = 4nm) of the molecule in his electron microscopy studies, however the angle of attachment to the long axis was seen to be more acute ie. 45°. More interestingly, however, the author also found that after exposure of the fibrinogen molecules to millimolar calcium, each terminal domain took on the form of two contiguous equal-sized spheres (diameter = 4nm). Thus, in the absence of calcium, the material in the subdomains was found to partially overlap onto each other whereas in the presence of millimolar

calcium the material in each subdomain remains seperate, resulting in two spherical globules. Consequently the overall effect of calcium on the D domain seems to be the dissociation or unfolding of the two comprising subdomains.

Mihalyi and Donovan (1985) also reported a calcium-induced conformational change within the D domain, as a result of their findings from calorimetric studies. The authors suggested that there must be at least two different kinds of unfolding units in the D nodule.

The enhancing effect of calcium on the fibrin polymerization process can be explained in part by this conformational change in the D domain leading to an increased affinity of polymerization sites within this domain for corresponding complementary sites in the E domain.

Doolittle (1973) proposed that the release of fibrinopeptides from the central domain allows intermolecular contact between the latter and the terminal domains of other molecules during polymerization. In 1978 Laudano and Doolittle found that short peptides beginning with the sequence Gly-Pro-Arg (glycine-proline-arginine), corresponding to the first three amino acids of fibrin α-chains exposed by release of fibrinopeptide A, can bind to fibrinogen and prevent the polymerization of fibrin monomers. The same authors also showed (1980) that peptides beginning with the corresponding β-chain sequence, Gly-His-Arg-

Pro (His = histidine) also bind to fibrinogen, albeit at seperate sites, but do not prevent polymerization. Gly-Pro-Arg-Pro binds to both sets of sites, although less tightly to the β-chain type. Both types of peptide were found to bind to the fragment D portions of fibrinogen.

Further studies by Laudano and Doolittle (1981) into the influence of calcium on the binding of fibrin amino terminal peptides to fibrinogen showed that the affinity of the amino terminal tetrapeptide of the β-chain of fibrin for fibrinogen increases in the presence of calcium; conversely no increase in the affinity of the corresponding α-chain peptides beginning with the sequence Gly-Pro-Arg was seen, although the number of binding sites did increase. (The latter was found to be due to binding of the α sequence to the β-chain site). The authors thus proposed that since the presence of calcium increases the affinity of Gly-His-Arg-Pro almost ten-fold, this indicates that the true physiological circumstances surrounding fibrin formation require the presence of calcium ions.

Furlan et al (1983) showed that the binding of calcium to low affinity sites on the molecule induces a conformational change in fibrin monomers. The ability of the peptide Gly-His-Arg to bind to fragment D thus increases, thereby accelerating the polymerization process.

The next investigation in these studies was concerned with determining which part (or parts) of the molecule is responsible for occlusion of the D domain. The calculation of experimentally and theoretically-derived values for incorporation of label into the Aα-chains as a function of calcium concentration were found to be in good agreement with each other. When these were compared to incorporation into the D domain it was found that the two parallel each other. This strongly suggests that certain regions of the Aα-chains are at least partly responsible for occlusion of the D domain.

The rise and fall of the incorporation into the D domain and Aα-chains in the first and intermediate phases (respectively) could be the result of intermolecular cross-linking occuring at this range of calcium concentrations. It is thus suggested that the Aα-chains dissociate intramolecularly as the calcium concentration is raised. The D domain is progressively uncovered, and both D and Aα regions incorporate more label. Intermolecular cross-linking similar to that suggested by Medved' et al (1986) can then occur, as a consequence of which both Aα-chain and D domainal regions are occluded. This results in a decrease in the radioactivity incorporated by both regions and the degree of incorporation levels off at this stage.

This sequence of events which is seen to occur as the calcium concentration is raised also correlates well with the observed conformational change in D. As seen previously, the

proposal presented in this study, ie. that the Aα-chains act as 'antennae' in fibrin formation, agrees with that of Medved' et al (1986). However from the results presented here it is further suggested that calcium ions not only trigger the dissociation of the Aα C-terminals, but also effect a conformational change in the D domain. Thus, as Aα intermolecular bonds are formed, conformational changes in the terminal domains occur. This renders the D domains more accessible and prone to intermolecular contacts, such as those that are made in the polymerization of fibrin monomers to protofibrils ie. DD-long and DE-stag contacts.

The presence of calcium is known to accelerate the formation of a fibrin clot from fibrinogen in the presence of thrombin. Thus, by extension, calcium-dependent conformational changes in the Aα C-terminals and D domains presented in this study may be at the basis of some of the events occuring in fibrin polymerization. It is thus suggested that calcium plays a role in stabilizing the fibrinogen molecule in a conformation that is favourable for its function in polymerization. However for these events to be linked directly to fibrin formation, fluctuations in the calcium concentration must occur in the microenviroment surrounding the forming fibrin clot. As seen previously, the amount of free calcium ions present in blood plasma is 10^{-3}M; the dependence on calcium for many of the events occuring in the coagulation cascade (Figure 1-1) has also been shown. When the wall of a blood vessel is transected, the collagen in the

vascular wall is exposed to the blood, and platelets immediately adhere to the exposed collagen fibres. These platelets then release calcium, 5-hydroxytryptamine, ADP, ATP and pyrophosphate simultaneously. If platelet calcium was equally distributed throughout the cell, its concentration would be approximately 30mM (Mills and Macfarlane, 1976); thus it is quite possible that the release of such calcium stores could cause fluctuations in the calcium concentration of the microenviroment immediately surrounding the forming blood clot, thus creating favourable conditions for calcium-dependent conformational changes in fibrinogen to occur.

It is interesting to note that this proposed mechanism can be paralleled to that which has been suggested for the activation of factor XIII at the site of clot formation (Curtis et al, 1974). As seen earlier, Credo et al (1978, 1981) reported that fibrinogen enhances the generation of fibrinoligase activity by lowering the calcium requirement of the thrombin-modified factor XIII zymogen (factor XIII') from 6 - 10mM, to physiological calcium concentrations. Thus at normal plasma concentrations of free calcium, thrombin and calcium alone cannot activate the enzyme, - fibrinogen is also required. However Curtis et al (1983) suggested that it is feasible for thrombin and calcium alone to activate factor XIII at the site of coagulation due to the elevated calcium concentration resulting from this ion's release by platelets.

Calcium was not the only divalent ion whose effects on the structure of fibrinogen were investigated in this study: magnesium and sodium chloride were also compared to calcium chloride. In Godal's (1960) studies on the effect of the anti-coagulant EDTA on human fibrinogen, the author reported that all effects of EDTA could be corrected by calcium while magnesium was much less effective. Marguerie et al (1977) showed that whereas the three high affinity binding sites of fibrinogen are specific for calcium, the low affinity sites are eliminated in the presence of 10^{-2}M magnesium chloride. The authors suggested that these are not specific and are due to weak interactions.

Cross-linking results showed that although magnesium does have some effect on the unfolding of the molecule's conformation, this does not occur to the same extent as that seen to result in the presence of calcium. This was also reflected in photolabelling experiments where higher magnesium concentrations were needed to bring about effects similar to those brought about by calcium. It was seen that within the range of magnesium concentrations investigated (ie. equivalent to those of calcium used), only phase I and the intermediate phase resulted. In the presence of calcium phase I occurs over the 0 - 2mM range whereas this is extended to 5mM in the presence of magnesium chloride. Thus higher magnesium concentrations are required to produce equivalent calcium effects; consequently these appear to be 'delayed' in magnesium. The conformational changes resulting in magnesium are very

likely to be due to the divalent ion binding to low affinity sites on fibrinogen, as reported by Marguerie et al (1977). However from the results seen in this study it is suggested that these sites do have a certain degree of specificity for calcium since the latter ions are more effective in bringing about conformational changes in the molecule compared to magnesium ions.

The effect of sodium chloride was also compared to that of calcium and magnesium chloride at an equivalent range of ionic strengths. From both cross-linking and photolabelling results it was apparent that sodium chloride has a negligible effect on the conformation of fibrinogen.

Thus, in this study, the two techniques of photosensitized radioactive surface labelling and photosensitized cross-linking have been developed and have also been shown to have definite applications in their use as probes into the conformation of fibrinogen. The results derived using these methods primarily support the view put forward by various authors that fibrinogen is a dynamic molecule having a flexible conformation and that the conformation adopted is dependent on solvent composition. Calcium concentration in the millimolar range is particularly significant and consequently so is the saturation of low affinity calcium binding sites which may well have a regulatory function. It is therefore suggested that fibrinogen is a dynamic isomer, with the predominant form at any one time being

dependent on certain physiological parameters, one major factor being calcium concentration. A possible suggestion concerning this point is that this change in the isomeric form of the molecule, which is under the control of physiological parameters, may be due to fibrinogen conforming to a specific structure where it becomes more feasible for a particular enzyme to act, according to the body's requirements at that particular point in time.

Experimentally, two extreme conformations have been demonstrated, - a closed compact structure at low calcium concentrations compared to a more open one at higher calcium concentrations. More importantly, however, the methods used also show the subtle changes which occur within the molecule as the calcium concentration is raised, changes which may be more significant physiologically. The most significant of these are the effects of calcium on the C-terminal parts of the Aα-chains and the D domains.

CHAPTER FIVE

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